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14. ABSTRACT
The goal of this Translational Pilot Award proposal is to create the basis for a clinical trial for JO-1 in combination with chemotherapy in patients with progressive ovarian cancer. Toward this goal we will perform i) detailed safety studies in DSG2 transgenic mice, ii) study the effect of JO-1 in ovarian cancer models, and iii) test combinations of JO-1 with major chemotherapy drugs. We report the following progress: 1) A manufacturing protocol for JO-1 at large scale has been established. 2) There were no adverse effects after intravenous injection of JO-1 in DSG2 transgenic mice. 3) JO-1 has favorable pharmacokinetics parameters. 4) The effect of JO-1 on normal tissues is minimal because DSG2 is trapped in epithelial junctions in normal tissues and not accessible to intravenously injected JO-1. 5) JO-1 enhances Doxil chemotherapy in xenograft models of ovarian cancer and in mouse models with syngeneic tumors.

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INTRODUCTION

The goal of this Translational Pilot Award proposal is to create the basis for a clinical trial for JO-1 in combination with chemotherapy in patients with progressive ovarian cancer. Toward this goal we will perform *i)* detailed safety studies in DSG2 transgenic mice, *ii)* study the effect of JO-1 in ovarian cancer models, and *iii)* test combinations of JO-1 with major chemotherapy drugs.

BODY

Task 1.1: GLP manufacturing of JO-1.

A manufacturing protocol for JO-1 at large scale has been established. The generation and characterization of a master *E.coli* cell bank has been completed. A pilot lot of JO-1 for toxicity and efficacy studies (Tasks 1.2., 2 and 3) and has been produced under GLP conditions by the FHCRC Biologics Manufacturing Core Laboratory. This lot met cGMP quality control standards defined by the FDA in a pre-IND meeting. We demonstrated that after 9 months of storage at -80^{o}C, JO-1 did not lose activity (test ongoing). After consultation with the FDA, the following *potency assays* of the cGMP product were established: *DSG2 binding assay:* The clinical function of the product is to bind to DSG2 triggering intracellular signaling. Binding of JO-1 to DSG2 was measured by ELISA. Rabbit polyclonal antibodies against DSG2 were used for capture followed by recombinant DSG2, the cGMP JO-1 lot/reference sample, mouse mAb against the Ad3 fiber knob, and anti-mouse IgG-HRP. *PEG permeability assay*: A total of 5x10^{5} T84 cells were seeded on 12 mm transwell inserts and cultured for 20 days until the transepithelial electrical resistance (TEER) remained constant, i.e. tight junctions have formed. The cells were exposed to JO-1 for 15 min at room temperature. 1 μCi of [^{14}C] polyethylene glycol-4000 (MW: 4,000Da) was added to the inner chamber. Medium aliquots were harvested from the inner and outer chambers and measured by a scintillation counter. Permeability was calculated as described elsewhere (11). JO-1 preparations are considered to be functionally potent if they increase PEG permeability >3-fold within 30 min.

Task 1.2: Toxicology studies in DSG2 transgenic mice.

There were no adverse effects or critical abnormalities found in hematologic and serum chemistry parameters or histopathological studies of tissues after intravenous injection of JO-1 (2 to 10 mg/kg) into hDSG2 transgenic mice (with or without pre-existing anti JO-1 antibodies. We observed a mild lymphocytopenia and intestinal inflammation that subsided by day 3 after injection. We speculate that the favorable safety profile of JO-1 is due to the fact that hDSG2 in tissues other than the tumor and a subset of epithelial cells in the intestine/colon is not accessible to intravenously injected JO-1.

Task 2.1: JO-1 biodistribution in DSG2 transgenic mice.

The hDSG2 transgenic mouse model was also used to obtain biodistribution and pharmacokinetics data for JO-1. These studies showed a serum half-life of 6 hours and hDSG2-dependent accumulation of JO-1 in epithelial cells of the small intestine and colon (1). We also found hDSG2 dependent binding of JO-1 to peripheral blood lymphocytes and hDSG2-independent uptake by liver, spleen and lymph node macrophages.

Task 2.2.: Kinetics of JO-1 and QD-labeled IgG accumulation in tumors. Instead of using quantum dot labeled IgG we studied the effect of JO-1 mediated junction opening in epithelial tumors and tissues of hDSG2 transgenic mice by measuring the uptake of PEG-modified gold nanoparticles (AuNP) with defined diameters of 35 and 120nm (Fig.1A). Notably, the average diameter of most encapsulated chemotherapy drugs is 100nm. We produced Au-NP and confirmed their size by dynamic light scattering. The biodistribution of AuNPs in tissue samples was quantified by inductively coupled plasma mass spectrometry (ICP-MS) at the Environmental Health Laboratory at the University of Washington (Seattle, WA). This method is more sensitive than the imaging of QD-labeled proteins. So far, we have measured the Au-NP amount in tumors (Figs.1B and C). Studies on other tissues are ongoing. In tumors, we found that the accumulation of 120nm Au-NP is at least 3 orders of magnitude less efficient than the accumulation of 35nm particles. The amount of 120nm AuNP per gram tumor tissue increased >10-fold when mice were pre-injected with JO-1. We are currently analyzing the AuNP penetration on tumor sections using transmission electron microscopy. We expect that the studies with AuNP will further corroborate that epithelial junctions represent a barrier to intratumoral accumulation of larger drugs) and that this can in part be overcome by JO-1.

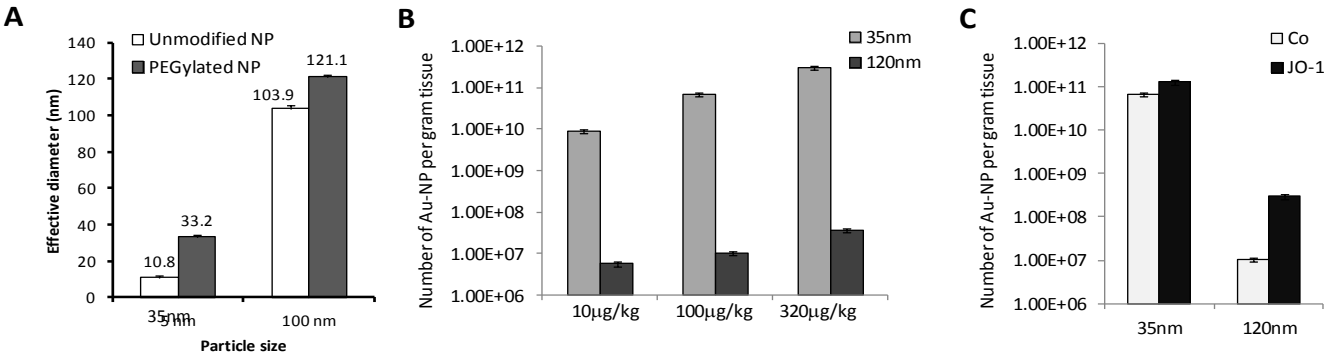

Figure 1. Accumulation of differently sized PEGylated gold-nanoparticles (Au-NP) in tumors. A) *Particle sizing of gold nanoparticles by dynamic light scattering (DLS) before and after modification with $mPEG_{5000}$-SH. Data are presented as mean ± standard deviation, N = 6. 5 nm were from Ted Pella, Redding, CA. 100nm particles were from Nanopartz, Inc., Loveland, CO.* ***B)*** *hDSG2 transgenic mice with syngeneic mammary fat pad MMC-hDSG2 tumors (800-1000mm^3) were intravenously injected with Au-NP at different doses. Six hours later blood was flushed from the circulation and tumors were harvested. Shown are the numbers of Au-NP particles per gram tumor. N=3.* ***C)*** *hDSG2 transgenic mice with established tumors were intravenously injected with PBS (Co) or JO-1 (2mg/kg) followed by an intravenous injection of Au-P at a dose of 100ug/kg. Tumors were harvested 6 hours later. N=3*

Task 2.2. c-1 and c-2: Studies on the activation of EMT pathways and junction opening in vivo are ongoing.

Task 2.3. Test whether JO-1 can facilitate existing immune responses in DSG2 transgenic mice bearing syngeneic tumors.

Using an immunocompetent hDSG2-transgenic mouse tumor model, we showed that JO-1 injection reduced the number of intratumoral Tregs, which in turn allowed pre-existing anti-tumor CD8 T-cells to control tumor growth (Fig.2). We used hDSG2 transgenic mice and syngeneic TC1 cells that expressed human DSG2. TC1 cells express HPV E6 and E7 and trigger E7-specific T-cells in C57Bl/6 mice. However, these tumor-specific T-cells are unable to control tumor growth and 100% of tumor-bearing animals reached the study endpoint by day 20. Two injections of JO-1 into tumor-bearing mice at days 6 and 14 resulted in complete tumor regression in 60% of mice (Fig.2A). This effect was due to an increased number of E7-specific T-cells (Fig.2B) and could be blocked by systemic depletion of CD8 cells. Importantly, we also found in flow cytometry analyses of tumor-infiltrating leukocytes that the number of Tregs (CD25+/FoxP3+) was about 6-fold less in JO-1 treated animal tumors compared to PBS injected animals (Fig.2C).

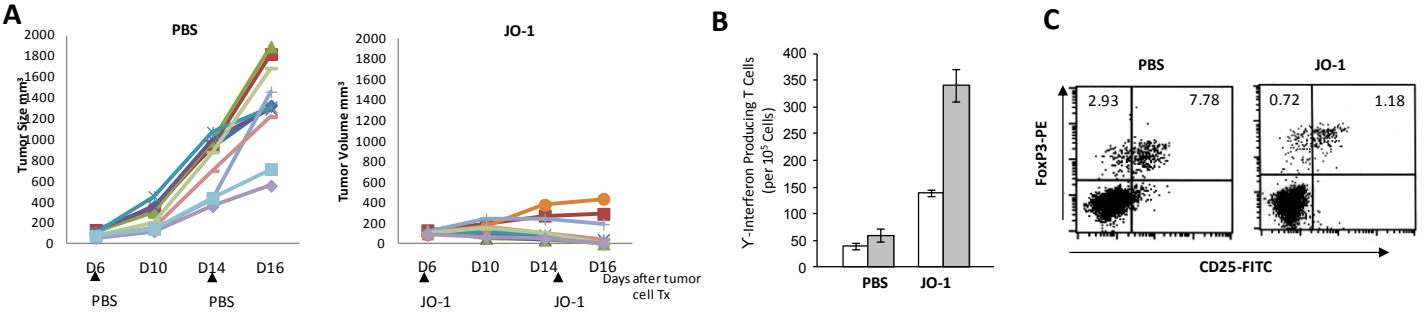

Figure 2. JO-1 facilitates anti-tumor T-cell responses. A) *hDSG2 transgenic mice with established subcutaneous syngeneic TC1-hDSG2 tumors were intravenously injected with PBS or JO-1 (2mg/kg) at days 6 and 14 after tumor cell transplantation and tumor volumes were measured. Each line represents an individual animal. N=10.* ***B)*** *Analysis of frequencies of IFNγ-producing E7 specific T-cells in the spleen 4 days after PBS or JO-1 injection. Shown are the frequencies of IFNγ producing T-cells specific to the HPV16 E749-57 carrying the H-2Db restricted peptide (RAHYNIVTF) (grey bars) or an unrelated control peptide (white bars). N=3 animals per group. The differences in the JO-1 group are significant ($p<0.05$).* ***C)*** *Analysis of intratumoral (FoxP3+) Tregs 4 days after PBS or JO-1 injection. Shown are representative flow cytometry data.*

We are currently studying JO-1 monotherapy in other syngeneic DSG2 transgenic mouse models.

Task 3.1. Test the co-therapies in xenograft tumor models.
We evaluated PEGylated liposomal doxorubin (PLD) in xenograft models with ovarian cancer cells (ovc316). Ovc316 cells are derived from a patient and closely model the heterogeneity and plasticity seen in tumors *in situ* (7). Mice with pre-established ovc316 tumors were intravenously injected with JO-1, followed one hour later by an intravenous injection of PLD (Doxil^{TM}). The ability of JO-1 to open up intercellular junctions increased the penetration and amount of PLD in tumors. Immunofluorescence analysis of tumor sections from JO-1/PLD treated animals showed PLD distributed over a greater distance from blood vessels (Fig.3A). More than 10-fold higher amounts of PLD per gram tumor tissues were measured by ELISA when PLD was combined with JO-1. The enhancing effect of JO-1 on PLD therapy was shown in a model with ovc316-derived mammary fat pad tumors (Fig.3B). Notably, the tumors were resistant to the PLD dose used in this study. The second model was an orthotopic model with intraperitoneal ovc316 tumors (Fig.3C). While all mice treated with PLD alone died by day 80 after tumor inoculation, the combination of JO-1 and PLD resulted in long-term survival (>160 days) of ~80% of animals.

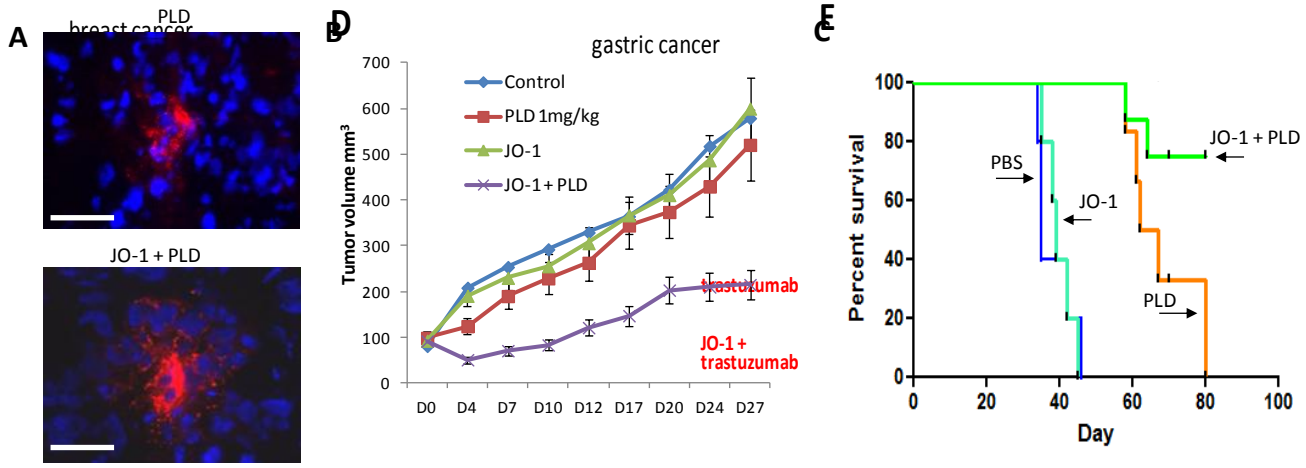

Figure 3. JO-1 enhances PLD therapy in an ovarian cancer model. A) *Better penetration and accumulation of PLD in tumors. Immunfluorescence analysis of tumor sections 2 hours after intravenous PLD or JO-1/PLD injection. PLD is stained red using antibodies that specifically bind to PEG in nanoparticles. The scale bars are 20 μm.* ***B)*** *Efficacy study/mammary fat pad model.Treatment was started when tumors reached a volume of 100 mm^3. Mice were injected intravenously with 2 mg/kg JO-1 or PBS, followed by an intravenous injection of PLD (1 mg/kg) or PBS one hour later. Treatment was repeated weekly.* ***C)*** *Survival study/intraperitoneal model. CB17-SCID/beige mice with intraperitoneal tumors derived from primary human ovarian cancer cells ovc316. Treatment was started at day 25 after tumor cell implantation and repeated weekly. Mice were injected intravenously with 2 mg/kg JO-1 or PBS, followed by an intravenous injection of PLD/Doxil (1 mg/kg) or PBS one hour later. Onset of ascites was taken as the endpoint in therapy studies. Shown is the survival of animals in a Kaplan Meier graph. n=10. p <0.001 for PLD vs JO-1 + PLD.*

Studies with other chemotherapy drugs are ongoing.
Our current University of Washington IACUC protocol number 3108-01 had to be renewed (mandatory 3-year renewal) in April 2013 and corresponding modifications had to be approved by the USAMRMC Animal Care and Use Review Office. This implied that we were not able to conduct animal studies from April to July 2013.

Task 3.2. Test lower doses of chemotherapeutics. Ongoing

Task 3.3. Test the co-therapies in immunocompetent DSG2 mice with syngeneic tumors
Tumor-specificity of JO-1 action: Because JO-1 does not bind to mouse cells (9), we generated human DSG2 (hDSG2) transgenic mice that expressed human DSG2 at a level and in a pattern seen in humans (9). Furthermore, we established syngeneic tumor cell lines (ID8, TC1, MMC (6)) with ectopic hDSG2 expression at levels seen in human tumors (1). In epithelial tissues of hDSG2 transgenic mice, hDSG2 is specifically localized to junctions (9). Furthermore, JO-1 triggers junction opening in hDSG2-expressing epithelial mouse tumor cells indicating that hDSG2 interacts with mouse signaling and cytoskeletal proteins thus overriding a potential function of mouse DSG2 in maintenance of junctions in transgenic mice. (Notably, there are no

mouse DSG2-specific antibodies available.) Using hDSG2 transgenic mice, we demonstrated that JO-1 predominantly accumulates in tumors (1). A number of factors could account for this finding, including: *i)* overexpression of hDSG2 by tumor cells, *ii)* better accessibility of hDSG2 on tumor cells, due to a lack of strict cell polarization compared to hDSG2-expressing normal epithelial cells, and *iii)* a high degree of vascularization and vascular permeabilty in tumors. Because of its preferential binding to and action on epithelial junctions of tumors, JO-1 appears to create a "sink" for therapeutic drugs in tumors, which decreases the levels and exposure of these drugs in normal tissues. A low level of hDSG2-specific JO-1 accumulation was also detected in the small and large intestine, specifically in intestinal epithelial cells that underwent cell division which potentially allowed for access to DSG2 (9).

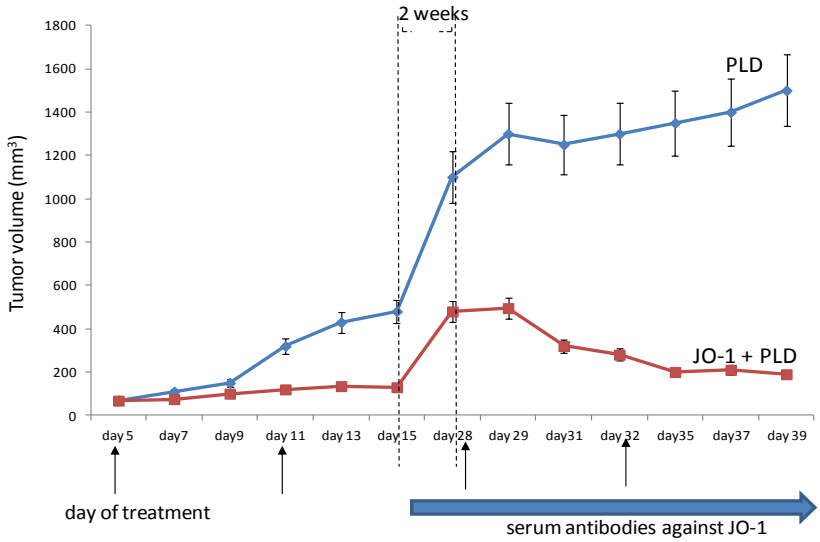

Figure 4. JO-1/PLD therapy in immunocompetent hDSG2 transgenic mice. *A total of 4x10^6 MMC-hDSG2 cells were injected into the mammary fat pad of hDSG2 transgenic mice. When tumors reached a volume of ~80 mm^3, JO-1 (2 mg/kg) or PBS was injected intravenously followed one hour later by PLD (i.v. 1.5 mg/kg). Treatment was repeated as indicated at day 5 and 11. Tumors were then allowed to regrow for about 2weeks. At this time, serum was analyzed for anti-JO-1 antibodies. Two more treatment cycles were performed at day 28 and day 32. N=5.*

Repeated JO-1/PLD treatment: JO-1 is an adenovirus-derived protein and therefore potentially immunogenic. This might not be a critical issue if JO-1 is used in combination with chemotherapy, which suppresses immune responses to foreign proteins. This expectation is supported by studies with oncolytic adenovirus vectors in which immunosuppression allowed for repeated vector application (2, 3, 8). Furthermore, we have demonstrated that JO-1 remains active *in vitro* and *in vivo* even in the presence of anti-JO-1 antibodies generated by JO-1 vaccination of mice (1). This may be due to the fact that JO-1 binds to DSG2 with a very high avidity thus disrupting potential complexes between JO-1 and anti-JO-1 antibodies. (Notably, JO-1 is a dimer of a trimeric fiber knob, which contributes to the picomolar avidity to DSG2 (10).) To test the potential for anti-JO-1 antibody responses to adversely affect the therapeutic effects of JO-1, we performed repeated injections of JO-1 in an immunocompetent hDSG2 mouse tumor model (Fig.4). After two treatment cycles of JO-1 and PLD, treatment was stopped and tumors were allowed to re-grow. The third and fourth treatment cycles were started on days 28 and 32, respectively. At the time of the third cycle, serum anti-JO-1 antibodies were detectable by ELISA in the JO-1/PLD treated group of animals. However, the antibody levels were about ~10-fold lower than in the group which received JO-1 without PLD. In both the third and fourth treatment cycles JO-1 had an enhancing effect on PLD therapy, demonstrating that JO-1 continues to be effective after multiple treatment cycles even in the presence of detectable antibodies.

JO-1 efficacy in an hDSG2 transgenic mouse model with spontaneous tumors. Transplanted tumors might differ phenotypically from tumors that arise spontaneously. We have therefore cross-bred hDSG2 transgenic mice with *neu*-transgenic (*neu*-tg) mice for 7 generations. Neu-transgenic mice overexpress the rat proto-oncogene *Neu* and develop spontaneous mammary tumors between 4 and 8 months of age (4, 5). The onset of tumor development was similar in double hDSG2/neu transgenic mice. The histology of spontaneous tumors in these mice reflects the main features seen in human breast cancer, specifically strong hDSG2 immunoreactivity in junctions between malignant cells (Fig.5A). As seen in the transplantable tumor models, JO-1 increased the efficacy of PLD treatment (Fig.5B).

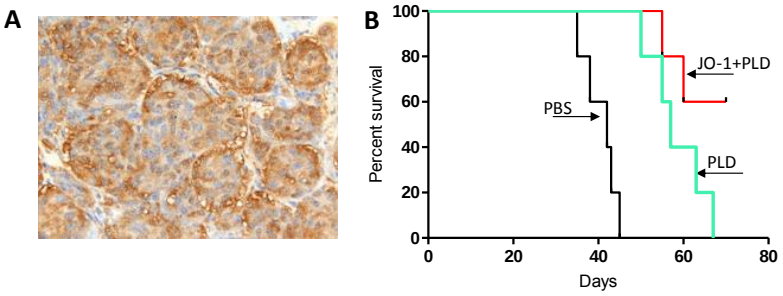

Figure 5. JO-1/PLD therapy in hDSG2/neu transgenic mice with spontaneous tumors. ***A)*** *DSG2 expression in spontaneous mammary tumors.* ***B)*** *Therapy study. As soon as mammary tumors were palpable, JO-1/PLD injection was started. Treatment was repeated weekly. The endpoint in the survival study was >20% loss in body weight due to cancer-related cachexia. N=4*

Studies with other chemotherapy drugs are ongoing.

KEY RESEARCH ACCOMPLISHMENTS

- A manufacturing protocol for JO-1 at large scale has been established.
- There were no adverse effects after intravenous injection of JO-1 in DSG2 transgenic mice.
- JO-1 has favorable pharmacokinetics parameters.
- The effect of JO-1 on normal tissues is minimal because DSG2 is trapped in epithelial junctions in normal tissues and not accessible to intravenously injected JO-1.
- JO-1 enhances Doxil chemotherapy in xenograft models of ovarian cancer and in mouse models with syngeneic tumors.
- JO-1 continues to be effective after multiple treatment cycles even in the presence of detectable antibodies.
- JO-1 facilitates pre-existing anti-tumor T-cell responses:

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations:

Beyer, I., Persson, J., Song, H., Cao, H., Feng, Q., Yumul, R., van Rensburg, R., Li, ZY, Berenson, R., Carter, D., Roffler, S., Drescher, C., Lieber, A. (2012) Co-administration of epithelial junction opener JO-1 improves the efficacy and safety of chemotherapeutic drugs
Clinical Cancer Research, 18(12):3340-51

Cao, H., Beyer, I., Lai, P-L., Disis, M., Lieber, A. 2013. Removal of physical barriers inside the tumor improves T-cell therapy. *Molecular Therapy*, 21:S5, presentation at the 16th annual meeting of the American Society of Cell and Gene Therapy

Beyer, I., Cao, H., Persson, J., Yumul, R., Lieber, A. 2013. A new epithelial junction opener for cancer therapy. *Molecular Therapy,* 21:S78, presentation at the 16th annual meeting of the American Society of Cell and Gene Therapy

Yumul, R., Cao, H., Richter, M., Gralow, J., Lieber, A. 2013. Affinity-enhanced epithelial junction openers improve cancer chemotherapy. *Molecular Therapy*, 21:S116, presentation at the 16th annual meeting of the American Society of Cell and Gene Therapy

Licenses applied/issued: none
Degrees obtained: none
Animal models: ovarian cancer model: DSG2 transgenic mice with ID8-DSG2 tumors
Funding applied for*:*

Competitive renewal, FHCRC Ovarian Cancer SPORE, Project 3: "A Combination Therapy of JO-1 and PEGylated Liposomal Doxorubicin in Ovarian Cancer Patients"

DoD Ovarian Cancer Teal Innovator Award "Targeting the tumor microenvironment to treat ovarian cancer."

With the preliminary data obtained in the context of the DoD award, additional funding for a clinical trial with JO-1 in ovarian cancer patients was obtained from Samyang Biotech (Seoul), Protein AI/INDRI (Seattle), and Chengdu Huasun Biotech (Chengdu).

Employment opportunities applied: one post-doctoral fellow (30% effort)

CONCLUSIONS

We included the data from the pre-clinical efficacy and safety studies obtained with JO-1 in mouse models of ovarian cancer into a pre-IND application for the use of JO-1 in patients with progressive, persistent or recurrent ovarian/fallopian tube cancer, who have previously received standard therapies. In a type B meeting, the FDA did not raise concerns that would delay the clinical development of the JO-1. Based on the FDA's recommendations, we now have clear guidelines for the next steps in completing the IND package.

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APPENDICES:

1) Pre-IND package submitted to the FDA
2) Written response from the FDA
3) Project 3 of Ovarian Cancer SPORE proposal

SUPPORTING DATA

N/A

PRE-IND MEETING INFORMATION PACKAGE FOR JO-1 / DOXIL COMBINATION THERAPY

(JO-1 epithelial tight junction opener with PEGylated liposomal doxorubicin, Doxil®)

Provided to: *Food and Drug Administration*
Center for Drug Evaluation and Research
Division of Biologic Oncology Products
Office of Oncology Drug Products
DDOP, HFD-150, 5901B
Ammendale Road, Beltsville, MD 20705-1266.

Mailing Address:

Rajesh Venugopal, MPH, MBA
Regulatory Health Project Manager
FDA WO-22 Room 6111
10903 New Hampshire Avenue
Silver Spring, MD 20993

For: *Type B Pre-IND Agency/Sponsor Teleconference*
Tuesday May 21, 2013, 2:00 PM EST

CDER Reference: *IND 117820*

Tuesday, April 02, 2013

TO: Rajesh Venugopal, MPH, MBA
Regulatory Health Project Manager
FDA WO-22 Room 6111
10903 New Hampshire Avenue
Silver Spring, MD 20993

FROM: Andre Lieber, MD / PhD
Department of Medicine
University of Washington
Seattle, WA 98195

RE: Pre-IND Type B Meeting (by teleconference) for:
PLD / JO-1 Combination Therapy

This pre-IND meeting information package includes information regarding: the proposed Phase 1 clinical trial; chemistry, manufacturing, and control information for junction-opening JO-1 biotherapeutic protein, pre-clinical safety and efficacy studies, and a proposed clinical study;

1) Product Name and Description

JO-1 is a small recombinant protein produced by fermentation in *E. coli* that targets desmoglein-2 (DSG2) in intercellular junctions. Preclinical studies demonstrate JO-1 binds to tumor tight junctions (TJs) and opens them, which enhances the entry of cancer therapeutics into tumors. JO-1 is a synthetic sequence with a dimerization domain, a linker domain and a DSG2 binding sequence derived from adenovirus type 3. The molecule forms dimers in solution. The predicted isoelectric point and molecular mass of the monomer are 8.8 and 27.8 kDa, respectively. **Doxil®** is a PEGylated liposomal form of the anthracycline topoisomerase inhibitor doxorubicin. This drug has been in numerous clinical trials, and it is approved for multiple indications including ovarian cancer after failure of platinum-based chemotherapy.

2) Chemical Name and Structure

PEGylated Liposome-encapsulated Doxorubicin (PLD) is available as Doxil®, Lipodox™, Caelyx™ and Myocet™. Doxil has been approved for the treatment of refractory ovarian cancer. Doxil is indicated for women with ovarian cancer, who have disease that is refractory to paclitaxel- and platinum-based chemotherapy regimens, which are current first-line therapies. Last year's Doxil shortage was caused by unplanned downtime due to equipment failures at the US manufacturer plants. These issues are being addressed by the manufacturer, and it appears that Doxil should be available in the future. To avoid similar problems with the availability of Doxil in the future, the Food and Drug Administration has allowed the import of Lipodox™ from Europe. Available data suggest that the activity and toxicity of Lipodox are comparable to Doxil. We will attempt to secure adequate supply of the Doxil preparation for our study and consider Lipodox in the event of shortage of Doxil.

JO-1: Recently, we developed a recombinant protein (JO-1) that transiently triggers the opening of intercellular junctions in epithelial tumors. This work is based on our finding that DSG2 is a high-affinity receptor for a number of human adenoviruses (Ad), including Ad serotype 3 [1,2]. JO-1 is a self-dimerizing, recombinant protein derived from the Ad3 fiber, which encompasses the binding protein for DSG2 [3] . JO-1 has a molecular weight of ~28 kiloDaltons (monomer) and binds with picomolar avidity to DSG2. It can be readily produced in *E.coli* and purified by affinity chromatography. *In vitro*, in polarized epithelial cancer cells, JO-1 triggered the transient opening of tight junctions [1,4], which increases the intratumoral permeability and penetration of large molecules.
JO-1 is formulated in phosphate buffered saline and stored frozen at -80^{o}C in 3 mL glass vials.

3) Proposed Indication for JO-1/Doxil for Infusion

The proposed indication for the investigational combination is recurrent or persistent epithelial ovarian, fallopian tube, or primary peritoneal cancer in patients who have failed standard therapies.
The purpose of the proposed Phase 1 clinical trial is to determine the safety, tolerability, and utility of JO-1 when administered in combination with Doxil in patients with progressive, persistent or recurrent ovarian/fallopian tube cancer, who have received standard therapies.

4) Purpose of the Meeting

The purpose of the meeting is to discuss the development of JO-1/Doxil for Infusion.

5) Specific Objectives/Outcomes Expected from the Meeting

The specific objectives of the meeting are:

- To determine if the proposed release test methods and specifications for the JO-1 therapeutic are acceptable to FDA.
- To determine if the proposed pre-clinical NHP safety studies are adequate to support the proposed clinical trial.
- To determine if FDA agrees with the design of the proposed Phase 1 clinical trial

6) Proposed Agenda

- Discussion of release test methods and specifications for the JO-1 therapeutic
- Discussion of pre-clinical safety studies
- Discussion of the clinical trial protocol

7) List of Specific Questions

- We were not able to generate JO-1 (with >95% purity) by methods other than His-tag/NTA agarose affinity purification. Are there concerns at the Agency regarding the use of a 6× histidinyl tagged therapeutic protein?

- The funds that we currently have available for the pre-clinical development of JO-1 are not sufficient to conduct a comprehensive GLP toxicology study despite the fact that the cost of doing these studies at the Washington State Northwest Primate Research Center (WaNPRC) at the University of Washington is 5-fold less than, for example, at a for-profit CRO such as BASi. Can the data from the non-GLP studies be used to supplement smaller GLP safety studies?

- Currently available data suggests that single agent JO-1 therapy is unlikely to have to a significant therapeutic effect. Our hypothesis, supported by our pre-clinical data, is that JO1 treatment will enhance the effectiveness of PLD therapy without increasing toxicity. Further compared to single agent JO1, JO1/PLD combination therapy is significantly less likely to lead to development of development of anti-JO1 antibodies that could impact therapy efficacy. As both the risks and benefits for study patients need to be considered we elected to design our trial to include JO-1/PLD combination therapy without single agent JO-1 treatment either as a run-in to the combination therapy or as a separate study. It is also notable that this study is sponsored by the FHCRC Ovarian Cancer SPORE with a relatively small budget. Are there concerns at the Agency regarding this trial design?

8) List of Sponsor Meeting Attendee

- André Lieber, MD/PhD, Professor, Department of Medicine, University of Washington, Seattle, WA 98195
  Dr. Lieber has developed the JO-1 technology and is performing further preclinical research

- Charles Drescher, MD, Fred Hutchinson Cancer Research Center, Seattle
  Dr. Drescher is a gynecologic cancer specialist. He will be the PI on the clinical trial

- Darrick Carter, PhD, Infectious Disease Research Institute, Seattle
  Dr. Carter is a biophysicist and will be addressing protein / interaction questions.

- Ron Manger, Ph.D., Director, Biologics Production / FHCRC
  Dr. Manger has been supporting production of JO-1 and will be addressing CMC questions

- Ron Berenson, MD, Compliment Corp. , Seattle
  Dr. Berenson will facilitate the interaction with the FDA and help in the preparation of the IND application.

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Full pathology report for NHP study

1. INTRODUCTION

Physical barriers for penetration of cancer drugs into tumors: Greater than 80% of all cancer cases are carcinomas, formed by the malignant transformation of epithelial cells. For most carcinomas, progression to malignancy is accompanied by a loss of epithelial differentiation and a shift towards a mesenchymal phenotype, *i.e.* epithelial to mesenchymal transition (EMT) [5]. EMT increases migration and invasiveness of many cell types and is often one of the conditions for tumor infiltration and metastasis. However, following invasion or metastasis, cells that have undergone the process of EMT can also revert to a well-differentiated epithelial phenotype [6]. In support, there exist numerous examples of advanced carcinomas showing that mesenchymal cells can regain characteristics of epithelial cells or undergo mesenchymal to epithelial transition (MET) [6]. One of the key features of epithelial tumors is the presence of intercellular junctions, which link cells to one another, and act as barriers to the penetration of molecules with a molecular weight of >400 daltons (Da) [7-9]. Several studies have shown that upregulation of epithelial junction proteins correlated with increased resistance to therapy, including therapy with the two major classes of cancer drugs - monoclonal antibodies and chemotherapeutics [10-12]. One of these junction proteins, desmoglein 2 (DSG2), is upregulated in malignant cells [13,14]. For example, we found consistently higher levels of expression of DSG2 in ovarian cancer cells than in the surrounding normal tissue or tumor stroma cells in more than 60 tumor biopsies analyzed [15]. Figure 1 shows immunohistological analyses of DSG2 on ovarian and breast cancer biopsies. Furthermore, mRNA profiling of ovarian cancer biopsies revealed overexpression of DSG2 mRNA. It is thought that the epithelial phenotype of ovarian cancer cells and their ability to form physical barriers protect the tumor cells from attacks by the host-immune system or from elimination by cancer therapeutics [6].

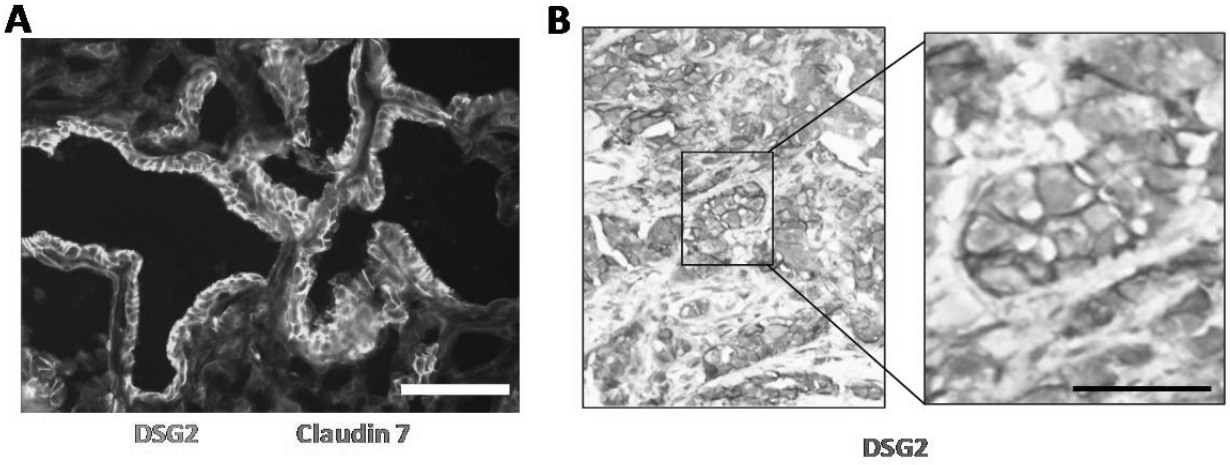


Figure 1. DSG2 is present in epithelial junctions of ovarian and breast cancer. **A)** Immunofluorescence analysis of an ovarian cancer biopsy. The sections were stained with antibodies against DSG2 (green) and the adherens junction protein claudin 7 (red). **B)** DSG2 immunohistochemistry on sections of a metastatic breast cancer lesion. DSG2 staining appears in brown. The scale bar is 20μm.

Tight junction openers: Various pathogens must first breach the epithelial barrier before gaining access to the body in order to initiate infection. Several mechanisms to disrupt junctional integrity have been developed by these pathogens, *e.g. Clostridium perfringens* enterotoxin removes claudins-3 and -4 from the TJ to facilitate bacterial invasion [16]. Also, Zona Occludens toxin (Zot) is produced by *Vibrio cholerae* strains and possesses the ability to reversibly modify intestinal epithelial TJs, granting the passage of

macromolecules through mucosal barriers [17]. Notably Cox *et al*. have shown that Zot increases the transport of drugs with low bioavailability (*e.g.* paclitaxel, doxorubicin, acyclovir, and cyclosporin A) up to 30 fold [18]. Additionally, oncoproteins encoded by human papillomavirus (HPV), human adenovirus, and human T-lymphotropic virus 1 (HTLV-1) can transiently open TJs by the mislocalization of the TJ protein ZO-1, thereby enhancing paracellular permeability in epithelial cells [19]. To date, however, there are no epithelial junction openers that are being used for cancer therapy. A number of chemical detergents, surfactants, calcium-chelating agents and phospholipids have been used to increase drug absorption through the gastrointestinal (GI) tract epithelium[20]. Recently, Kytogenics Pharmaceuticals, Inc. has developed a TJ opener based on chitosan derivatives. It is thought to act by electronegative forces applied to TJ proteins (http://www.kytogenics.com). However, all of these agents act indiscriminately to mechanically disrupt junctions and cannot be applied systemically without major toxic side effects.

Adenovirus serotype 3 derived junction opener JO-1: Human adenoviruses have been classified into seven species (A to G) currently containing 57 serotypes. We have recently reported that a group of human adenoviruses uses DSG2 as a receptor for infection [1]. Among DSG2-targeting viruses is serotype 3 (Ad3). Ad3 is able to efficiently breach the epithelial barrier in the airway tract and infect airway epithelial cells. This is achieved by the binding of Ad3 to DSG2, and subsequent intracellular signaling that results in transient opening of tight junctions between epithelial cells. We have capitalized on this mechanism and created a recombinant protein that contains the minimal structural domains from Ad3 that are required for opening of the intercellular junctions in epithelial tumors. This protein is called "junction opener 1" or "JO-1". JO-1 is a self-dimerizing recombinant protein derived from the Ad3 fiber [3]. JO-1 has a molecular weight of approximately 60 kiloDaltons (kDa). It can be easily produced in *E. coli* and purified by affinity chromatography. JO-1 binding to and clustering of DSG2 triggers an EMT that results in transient opening of epithelial junctions. We have shown in over 25 xenograft tumor models that the intravenous injection of JO-1 increased the efficacy of cancer therapies, including many different monoclonal antibodies and chemotherapy drugs, in a broad range of epithelial tumors. Further studies showed that the effective doses of chemotherapy can be reduced when the chemotherapy drugs are combined with JO-1. Finally, our studies have demonstrated that combining JO-1 with chemotherapy drugs markedly reduced the toxic side effects of chemotherapy. The application of JO-1 was safe and well-tolerated in toxicology studies carried out in human DSG2-transgenic mice and a limited number of macaques [1,4].

Phase clinical trial: The primary goal of this trial is to confirm the feasibility and safety of JO-1 when administered in combination with PEGylated liposomal doxorubicin (PLD) in patients with progressive, persistent or recurrent ovarian/fallopian tube cancer, who have received standard therapies. Three dose levels of JO-1 will be tested in combination with a fixed (standard) dose of PLD (40mg/m^2). Patients will be treated for 4 cycles (treatment given once every 4 weeks [standard schedule]) or until toxicity or tumor progression.

2. PROPOSED PHASE 1 CLINICAL TRIAL

2.1 Rationale for Study

2.1.1 Rationale for JO-1 in Combination Therapy

Need for improvement of PLD therapy: PLD is used to treat patients with ovarian cancer that progressed or recurred after platinum-based chemotherapy. Although a drug commonly used to treat recurrent ovarian cancer, response rates to PLD are low, the response duration is short, and the toxicity is significant. The overall response rate in a study with 239 patients was 19.7% (3.8% and 15.9% complete and partial response, respectively) (http://www.doxil.com/). Progression-free survival (PFI) was roughly 29 weeks and 9 weeks for patients with platinum sensitive and platinum resistant disease, respectively. A recent Phase 3 trial demonstrated a modest improvement in PFI (11.3 vs 9.3 months, p= .005) and no change in overall survival for PLD-carboplatin *vs*. paclitaxel/carboplatin combination chemotherapy in patients with recurrent platinum-sensitive ovarian cancer. Side effects of treatment include mucositis, hand-foot syndrome, myelosuppression, cardiotoxicity, and liver impairment. There is a significant risk of congestive heart failure when the total cumulative dose of doxorubicin HCl approaches 550 mg/m² although reduced cardiac toxicity with pegylated doxorubicin has been reported. When used as mono-therapy for recurrent epithelial ovarian cancer at doses of 50mg/m^2, roughly 30% of patients develop a grade 3 toxicity of some type. We decided to test JO-1 (for its first application in humans) in combination with PLD because it is has a major role in ovarian cancer therapy and there is a large body of experience with this drug. Further new ovarian cancer therapeutics are often tested in combination with PLD (see <ClinicalTrials.gov>).

The effective size of the PLD is 90nm. Given the large size, we postulate that PLD tumor penetration is a limiting factor for PLD therapy, and that the low response rate and undesired toxicities of PLD in ovarian cancer patients can be overcome by combining the drug with JO-1 treatment.

2.1.2 Rationale for Proposed Administration Protocol

JO-1 suspended in 200 ml of saline will be administered i.v. and PLD will be administered i.v. 60 minutes after completion of JO-1 infusion. JO1 opens epithelial junctions through binding to DSG2, DSG2 cleavage and induction of intracellular signaling. Our preclinical studies demonstrated that these events occur within one hour after intravenous infusion of JO-1. Furthermore, separating JO-1- and PLD infusions by one hour will allow us to assess for potential immediate side effects associated with JO-1 infusion.

Why not JO-1 alone? As outlined below, analysis of serum samples collected at diagnosis from >60 ovarian cancer patients did not reveal detectable antibodies against JOI-1. However, JO-1 infusion into patients that do not receive concomitant Doxil therapy, may trigger immune responses, specifically, antibodies against JO-1. Anti-JO-1 immune responses bear safety risks and might also affect the therapeutic effects. Because there is an increased risk and no clear therapeutic benefits to the patients, we decided not to include single agent JO-1 therapy in our trial design. . In this context, it is also notable that this study is sponsored by the FHCRC Ovarian Cancer SPORE with a relatively small budget.

2.2 Study Objectives and Design

2.2.1 **Objectives**

The primary goal of this trial is to confirm the feasibility and safety of JO-1 when administered in combination with PEGylated liposomal doxorubicin (PLD) in patients with progressive, persistent or recurrent ovarian/fallopian tube cancer, who have received standard therapies. Three dose levels of JO-1 will be tested in combination with a fixed (standard) dose of PLD (40mg/m^2). Patients will be treated for 4 cycles (treatment given once every 4 weeks [standard schedule]) or until toxicity or tumor progression.

Primary Objectives:

1. To determine the maximum tolerated doses (MTD) and dose limiting toxicities (DLTs) of single dose JO-1 administered in combination with PEGylated liposomal doxorubicin (PLD; Doxil®) at a standard dose (40 mg/m^2) and the associated DLTs based on adverse events that occur in cycle 1 for each of these combinations in women with recurrent or persistent, epithelial ovarian, fallopian tube or primary peritoneal cancer.

2. To examine the tolerability of the combination at the MTD of JO-1 assessed in combination with PLD 40 mg/m^2.

3. To determine recommended Phase 2 doses (RP2D) of JO-1 in combination with PLD.

Secondary Objectives/Translational Research Objectives:

1. To assess the biological effects of JO-1 in combination with PLD.
2. To assess the JO-1 and PLD pharmacokinetics in patients.

Exploratory Objectives:

1. To assess the anti-tumor activity of JO-1 when administered concomitantly with PLD.
2. To assess hypoxia parameters.
3. To assess anti-JO-1 and anti-tumor immune responses

2.2.2 **Design**

Intravenous infusion of PLD will start one hour after the completion of intravenous infusion of JO-1. The treatment cycle is repeated every 28 days for a total of 4 cycles or until disease progression or unacceptable toxicity. Patients will be entered in 3 dose cohorts of 3–6 patients each until the recommended Phase 2 dose (RP2D) is estimated. The starting and subsequent dose cohorts of JO-1 will be determined based on results from on-going pre-clinical NHP studies as well as the results of the clinical study.

Patients will be hospitalized and observed for 23 hours after each administration of JO-1/PLD and then evaluated in the outpatient clinic daily during the first 2 days after treatment and then weekly. Standard protocols for monitoring of patients treated with Phase I agents will be followed. Blood will be drawn before injection, 1, 2, 3, 4, 6 hours post-injection (p.i.), 12 hours p.i., 24 hours p.i., 48 hours p.i., 72 hours p.i. and then weekly. Standard safety parameters will be measured including clinical status, physical examination including vital signs, weight, hematologic parameters, serum chemistry studies, coagulation

studies and electrocardiograms.

JO-1 dose Range: In the NHP tox study we propose to test 0.6mg/kg and 2mg/kg. (0.6mg/kg in macaques = 2mg/kg in mice after allometric scaling*). The "high" dose in macaques (2mg/kg) was chosen since we do not anticipate going higher than this dose in any of our human clinical studies. For the trial in humans, we propose to test three JO-1 doses. Assuming the preclinical toxicology demonstrates adequate safety for the 0.6 mg/kg dose in non-human primates we will use that dose as a starting dose. The trial will be a dose escalation (1.0, 2.0 mg/kg) trial.

*Recent studies have documented that interspecies allometric scaling for proteins is equal to or superior to the accuracy of scaling for small molecule drugs [21].

2.3 Study Procedures

2.3.1 Schedule of Subject Evaluations

Patients will receive JO-1 via intravenous administration. The assigned dose of JO-1 will be suspended in 200ml of sterile saline, and administered intravenously at a rate of 2ml/minute, through a peripheral or central intravenous line. PLD (40mg/m^2) will be given 1 hour later. JO-1/PLD related toxicity/adverse events and clinical response to treatment will be evaluated as outlined in below. Patients will be treated for 4 cycles (treatment given once every 4 weeks). Monitoring as shown in Table 2 will be repeated for each cycle.

Table 2: Subject Evaluations. (The same procedures will be repeated for each treatment cycle.)

	Pre-Enrollment	Each Cycle	Day 1[#]-3 post	Wk 1 post	Wk 2 post	Wk 3 Post
Treatment (JO-1+/-PLD)		Day 0				
Clinical Analysis						
Clinical status/adverse events	X	X	X	X	X	X
Physical	X	X	X	X	X	X
EKG	X	X	X			
CT or MRI of Abdomen/Pelvis*	X					
Chest X-ray (CXR)**	X					
Blood collection (CBC,diff, PLTS; Chem 23,	X		X	X	X	X
CA125	X	X				
Urine analysis	X	X	X	X	X	X
Pregnancy Test (if not post-hysterectomy)	X					
Tumor biopsy (if possible)***	X					
Serum antibodies to JO-1****	X	X	X	X	X	X
Serum cytokines (5) ****	X	X	X	X	X	X
JO-1 in serum ****	X	X	X	X	X	X
Anti-JO-1 antibodies in serum****	X	X	X	X	X	X
PLD concentrations in serum****	X	X	X	X	X	X

[#] Day 1 follow-up evaluation and testing will be performed in hospital prior to discharge.

* Repeated every other cycle

**Repeated every other cycle if initially abnormal

*** Obtain tissue block from a diagnostic surgery or procedure. If patient undergoes biopsy (including para or thorocentesis for clinical indication during treatment we will attempt to collect tissue samples. This will be done through the POCRC clinical core protocols: to be used to perform immunohistochemistry for DSG2, and tissue markers of hypoxia;
**** to be done in PI's lab.

MUGA scans (Multi Gated Acquisition Scan) will be done before the start of cycle I and after 4 cycles of PLD to evaluate cardiac function.

2.3.2 **Subject Selection**

Patients with histologically or cytologically confirmed epithelial ovarian, fallopian tube or primary peritoneal cancer that is persistent or recurrent following primary treatment that includes a platinum and taxane compound (and up to two additional therapies). Patients who have received only one prior cytotoxic regimen (platinum based regimen for management of primary disease), must have a platinum-free interval of less than 6 months, or have progressed during platinum-based therapy, or have persistent disease after a platinum-based therapy.

Patients with the following histologic cell types are eligible: serous adenocarcinoma, endometrioid adenocarcinoma, mucinous adenocarcinoma, undifferentiated carcinoma, clear cell adenocarcinoma, mixed epithelial adenocarcinoma, transitional cell carcinoma, malignant Brenner's tumor, or adenocarcinoma not otherwise specified (N.O.S.). Patients with measurable or detectable disease are eligible. Measurable disease (defined as at least one lesion that can be accurately measured in at least one dimension) will be characterized using RECIST 1.1 criteria. Detectable disease is defined as not having measurable disease but has baseline values of CA-125 ≥ 2 x ULN.

Standard exclusion criteria will be employed. Additionally, patients cannot have received prior therapy with PLD or an anthracycline. Patients with clinically significant cardiovascular disease (uncontrolled hypertension, Myocardial infarction or unstable angina within 6 months of study enrollment, history of serious ventricular arrhythmia, New York Heart Association (NYHA) Class II or higher congestive heart failure) or patients with baseline ejection fraction < or = 50% as assessed by echocardiogram or MUGA will also be excluded. Finally, patients with pre-treatment anti-JO1 antibodies will be excluded.

2.3.3 **Clinical and Laboratory Evaluations**

Blood will be drawn before injection, 1, 2, 3, 4, 6 hours post-injection (p.i.), 12 hours p.i., 24 hours p.i., 48 hours p.i., 72 hours p.i. and then weekly. Blood samples will be used to evaluate JO-1 and PLD kinetics, immunology, and biologic effects per the schedule outline in table 1. Standard safety parameters will be measured including clinical status, physical examination including vital signs, weight, hematologic parameters, serum chemistry studies, coagulation studies and electrocardiograms. Potential toxicities and side effects of JO-1 injection could include *i)* gastrointestinal symptoms: nausea, vomiting, diarrhea, *ii)* leukopenia, *iii)* inflammation due to transient elevation of serum pro-inflammatory cytokines (IL-6, TNF-α, INF-γ), and *iv)* immunogenicity. Disease status will be assessed using clinical examination, imaging and serum CA125 levels.

2.4 Study Article

2.4.1 **PEGylated Liposomal Doxorubicin (DOXIL, Lipodox™; NSC #712227)**

Refer to the PLD package insert (Doxil, Lipodox™) for the most complete and current information on the following:

Formulation: PLD (doxorubicin HCl liposome injection) is supplied as a sterile, translucent, red liposomal dispersion in 5 mL (Lipodox only), 10 mL, or 30 mL glass, single-use vials. Each vial contains doxorubicin HCl at a concentration of 2 mg/mL.

Storage: Refrigerate unopened vials of PLD at 2°–8°C (36°–46°F). Avoid freezing. Prolonged freezing may adversely affect liposomal drug products; however, short-term freezing (less than 1 month) does not appear to have a deleterious effect on PLD.

Preparation: PLD doses up to 90 mg must be diluted in 250 mL of 5% Dextrose Injection, USP prior to administration. Doses exceeding 90 mg should be diluted in 500 mL of 5% Dextrose Injection, USP prior to administration. Aseptic technique must be strictly observed since no preservative or bacteriostatic agent is present in PLD. Diluted PLD should be refrigerated at 2°C–8°C (36°F–46°F) and administered within 24 hours.

- Do not mix with other drugs.
- Do not use with any diluent other than 5% Dextrose Injection.
- Do not use any bacteriostatic agent, such as benzyl alcohol.

PLD is not a clear solution but a translucent, red liposomal dispersion. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit. Do not use if a precipitate or foreign matter is present.

Procedure for Proper Handling and Disposal: Caution should be exercised in the handling and preparation of PLD. The use of gloves is required.

If PLD comes into contact with skin or mucosa, immediately wash thoroughly with soap and water.

PLD should be considered an irritant and precautions should be taken to avoid extravasation. With intravenous administration of PLD, extravasation may occur with or without an accompanying stinging or burning sensation, even if blood returns well on aspiration of the infusion needle. If any signs or symptoms of extravasation have occurred, the infusion should be immediately terminated and restarted in another vein. PLD must not be given by the intramuscular or subcutaneous route.

PLD should be handled and disposed of in a manner consistent with other anticancer drugs.

Adverse Effects: Consult the PLD package insert for the most current and complete information.

Supplier: Commercially available from Ortho Biotech Products, LP Raritan, NJ (DOXIL) and Caraco Pharmaceutical Laboratories Ltd, Detroit, MI (Lipodox).

Consult the American Hospital Formulary Service Drug Information guide, Facts and Comparisons, or the package insert for additional information.

Lipodox is comparable to Doxil. We will attempt to secure adequate supply of the Doxil preparation for our study and consider Lipodox in the event of shortage of Doxil.

JO-1: Recently, we developed a recombinant protein (JO-1) that transiently triggers the opening of intercellular junctions in epithelial tumors. This work is based on our finding that DSG2 is a high-affinity receptor for a number of human adenoviruses (Ad), including Ad serotype 3 [1,2]. JO-1 is a self-dimerizing, recombinant protein derived from the Ad3 fiber, which encompasses the binding protein for DSG2 [3] . JO-

1 has a molecular weight of ~28 kiloDaltons (monomer) and binds with picomolar avidity to DSG2. It can be readily produced in *E.coli* and purified by affinity chromatography. *In vitro*, in polarized epithelial cancer cells, JO-1 triggered the transient opening of tight junctions [1,4], which increases the intratumoral permeability and penetration of large molecules.
JO-1 is formulated in phosphate buffered saline and stored frozen at -70°C in 5 mL glass vials.

Combination Therapy: The proposed indication for the investigational combination is recurrent or persistent epithelial ovarian, fallopian tube, or primary peritoneal cancer in patients who have failed standard therapies. The purpose of the proposed Phase 1 clinical trial is to determine the safety, tolerability, and utility of JO-1 when administered in combination with Doxil in patients with progressive, persistent or recurrent ovarian/fallopian tube cancer, who have received standard therapies.

2.5 Criteria for Interrupting the Trial

2.5.1 Rules for Discontinuing Study Injections in an Individual Subject

The development of a DLT defined as any grade 3 or 4 non-hematologic toxicity as evaluated by the NCI Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0, Grade 4 neutropenia lasting >7 days or Grade 4 febrile neutropenia occurring during cycle 1 of therapy that is possibly, probably, or definitively related to JO-1 administration or any Serious Adverse Events (SAE) whether related to study drug or not will be considered indication for stopping study drug in an individual patient. Standard protocols will be followed to ensure timely reporting of all adverse events. Exclusions to the above definition of DLT include Grade 3 hand-foot syndrome, fatigue and nausea, vomiting or diarrhea or metabolic abnormalities lasting less than 48 hours as these occur not infrequently with PLD therapy in the study population.

2.5.2 Rules for Suspension of the Entire Study

Standard Phase 1 3+3 design dose escalation and stopping rules will be followed. A 3-step dose escalation strategy will be employed. For each cohort, up to 3 evaluable patients will be enrolled and treated at the assigned dose level. There will be a separation of at least 3 days before the start of treatment of each patient. Safety data through Cycle 1 for all 3 patients must be reviewed before enrollment of the next cohort can be considered. If none of the 3 patients experience a dose limiting toxicity (DLT) during Cycle 1, dose escalation will proceed, and 3 new patients will be enrolled at the next dose level. If 1 of 3 patients in a cohort experiences a DLT during Cycle 1, the cohort will be expanded, and up to 3 additional patients treated at that dose level before JO-1 dose escalation can proceed. If a total of 1 of 6 patients experiences a DLT during Cycle 1, dose escalation may continue. If 2 or more patients in a cohort experience a DLT in Cycle 1, no additional patients will be treated at that dose of JO-1 and the previous dose level will be considered: if six patients have been treated at that dose level, it will be considered the maximum tolerated dose (MTD); if three patients have been treated at that dose level, up to three more will be treated at that dose level. A total of six patients must be treated at a dose level, with 2 patients experiencing DLTs, for a dose level to be declared the MTD.

2.6 Statistical Considerations

This trial has the primary aim of assessing the frequency and severity of toxicities including DLTs related to treatment with JO-1 in combination with PLD and to identify the MTD and a recommend JO-1 dose

for testing in a Phase II trial. A traditional 3+3 study design is employed allowing dose escalation until 2 out of 6 (i.e. > or = 33%) of participants experience a DLT. A 3-step dose escalation scheme is proposed with the highest dose exceeding the range where therapeutic efficacy is expected. If MTD is not reached at the highest dose cohort, this dose will be defined as the recommended phase II dose (RP2D) otherwise the dose level immediately below the MTD will be considered the R2PD. Tumor response is an exploratory endpoint and will be asses as described in section 2.6.1. Descriptive analysis will be given for the pharmacokinetic parameters of JO-1 and PLD.

The study is not designed to have the power to allow for formal comparison of anti-tumor efficacy response. However, the event rate (complete response, partial response, stable disease, progressive disease and time to progression) will be reported. Informal comparison will be made to historical data from other studies in EOC patients treated with PLD. Designing a trial that will have statistical power to identify a therapeutic effect will require more patients. (For example, in order to predict with a 90% confidence interval that at least 50% of all patients injected show a therapeutic effect in phase II (efficacy) testing, 70% of 20 to 30 subjects would need to have a response in phase I testing.) Larger number of patients per arm would also allow us to assess rare toxicity with more confidence. Clearly, we will increase the number of patients if additional funding for the trial becomes available.

2.6. Biological Activity Analyses

Tumor response: Although not a primary endpoint of the study tumor response will be assessed using clinical examinations, serum levels of CA125 prior to each cycle of therapy and imaging (CT or MRI) prior to every other cycle of therapy and at the end of the treatment period. For patients with measureable disease Standard Response Evaluation Criteria In Solid Tumors (RECIST) criteria will be applied to assess a therapeutic response. If there are numerous (>5) measurable lesions at baseline, up to five lesions (with no more than 2 lesions per organ) will be chosen for measurement before the patient is entered into the study. The remaining measurable lesions will be considered unevaluable for tumor response, but remain evaluable for disease progression and safety. For patients with non-measurable disease, CA-125 will be used to define biochemical response and progression according to the following guidelines (patients must have elevated CA125 at baseline). If the patient's CA-125 normalizes (i.e., ≤ 1 x ULN) after treatment, then the patient will be considered to have a biochemical response. A patient will be considered to have biochemical progression if CA-125 is ≥ 2x nadir value on two occasions at least a week apart.
The best overall response is the best time point response recorded from the start of the treatment until disease progression or study end. The duration of overall response is measured from the time measurement criteria are met for CR or PR (whichever is first recorded) until the first date that recurrent or progressive disease is objectively documented.

Tumor hypoxia: The finding that JO-1 decreases hypoxia is relevant for cancer therapies especially radiotherapy which is often inefficient in hypoxic environments [22]. Recent data suggest that tumor hypoxia can be assessed non-invasively using PET imaging strategies. However PET imaging (which is not approved for use in monitoring ovarian cancer) is outside the bounds of our study. Consequently we will evaluate the effect of JO-1 on tumor hypoxia indirectly via serial measurements of serum markers (VEGF) and tissue markers (HIF-1, GLUT-1 and CAIX) in pre and post treatment biopsies when available.

2.7. Immunogenicity Analyses

JO-1 serum concentrations: Serum samples will be stored at -80°C. JO-1 concentrations will be measured by ELISA using rabbit polyclonal anti-Ad3 fiber antibodies for antigen capture and anti-6-His tag mAbs/anti-mouse IgG-HRP) for detection [23]. The detection limit of this ELISA is 50pg/ml.

Serum anti-JO-1 antibodies: Serum samples will be stored at -80°C. Anti-JO-1 antibodies will be measured by ELISA. Plates will be coated with rabbit polyclonal anti-Ad3 fiber antibodies, followed by recombinant JO-1, human serum samples (1:2 to 1:1000 dilution), and either anti-human IgG-HRP or anti-human IgM-HRP.

Pro-inflammatory cytokine analysis: Serum samples will be stored at -80°C. Cytokine will be performed with BD Cytometric Bead Array (CBA) Human Soluble Protein Flex Set (Becton Dickinson, Franklin Lakes, NJ, US) according to manufacturer's instructions. FCAP Array (TM) v1.0.1 software will be used for data analysis.

Assessment of tumor and JO-1 specific immunity by ELISPOT: We found that JO-1 injection into immunocompetent mice with syngeneic DSG2-positive tumors increased the frequency of tumor-specific T-cells in the tumor, but also in the periphery, e.g. the spleen (see section 4.5.8.). This observation warrants the analysis of anti-tumor T-cells in the peripheral blood of patients.PBMCs will used to evaluate the induction of tumor and adenovirus specific immunity following treatment as reported [24,25]. PBMCs will be isolated by Percoll gradient. Cells will be frozen in CTL-CryoABCTM serum-free media (Cellular Technology Ltd. Cleveland, Ohio). ELISPOT will be performed according to MABtech manufacturer instructions (h-INF-gammaELIPOST PRO 10 plate kit). For JO-1 ELISPOT, cells will be stimulated with the JO-1 protein. For the anti-tumor response survivin (BIRC5 PONAB), pool of CEA+Ny-ESO-1, pool of c-Myc +SSX2, MAGE-3 and WT-1 peptides were used (ProImmune). Of note, no pre-stimulation of PBMCs will be done in order to avoid artificial or incorrect signals and to ensure adequate viability of cells which might be compromised during prolonged culture.

2.8. Safety Analyses

Patients should be monitored for study-drug related toxicity as outlined in Table 1 above. Study investigator should carefully assess all treatment-associated toxicities and, whenever possible, determine if they can reasonably be attributed to PLD alone or the treatment regimen based on toxicity grades (Table 2). Thus far diarrhea is the only observed toxicity of JO-1 in pre-clinical studies. PLD is associated with a number of toxicities including hand-foot syndrome, myleosuppression and stomatitis. PLD dose modification for these toxicities are as described below.

PLD Dose adjustment guidelines for hematological toxicities (ANC and Platelets), hand-foot syndrome are as follows:

Hematological toxicity:

Grade 1: (ANC cells/mm^3) (PLTS cells/mm^3) 1 1,500–1,900 75,000–150,000; Resume treatment;
Grade 2: 1,000–< 1,500 50,000–< 75,000 Wait until ANC ≥ 1,500 and platelets ≥ 75,000; redose with no dose reduction
Grade 3: 500–999 25,000–< 50,000. Wait until ANC ≥ 1,500 and platelets ≥ 75,000; redose with no dose reduction

Grade 4: < 500 < 25,000. Wait until ANC ≥ 1,500 and platelets ≥ 75,000; redose with 25% dose reduction (excluding G4 toxicity lasting >7d or associated with febrile neutropenia in Cycle 1. This will be considered a DLT and patients will come off study.)

Table 2: Definition of toxicity grades

Toxicity Grade	Dose Adjustment
1: painless ulcers, erythema, or mild soreness	**Redose unless patient has experienced previous Grade 3 or 4 toxicity.** If so, delay up to 2 weeks and decrease dose by 25%. Return to original dose interval.
2: painful erythema, edema, or ulcers, but can eat	**Delay dosing up to 2 weeks or until resolved to Grade 0-1.** If after 2 weeks there is no resolution, PLD should be discontinued. If resolved to Grade 0–1 within 2 weeks and there was no prior Grade 3-4 stomatitis, continue treatment at previous dose and return to original dose interval. If patient experienced previous Grade 3–4 toxicity, continue treatment with a 25% dose reduction and return to original dose interval.
3: painful erythema, edema, or ulcers, and cannot eat	**Delay dosing up to 2 weeks or until resolved to Grade 0–1.** Decrease dose by 25% and return to original dose interval. If after 2 weeks there is no resolution, PLD should be discontinued. Grade 3 toxicity in Cycle 1- off study.
4: requires parenteral or enteral support	**Delay dosing up to 2 weeks or until resolved to Grade 0–1.** Decrease dose by 25% and return to PLD original dose interval. If after 2 weeks there is no resolution, PLD should be discontinued. Grade 4 toxicity in Cycle 1- off study.

Hand-foot syndrome:
Grade 1: mild erythema, swelling, or desquamation not interfering with daily activities – no delay
Grade 2: erythema, desquamation, or swelling not precluding normal physical activities; small blisters or ulcerations <2 cm in diameter ; **Delay dosing up to 2 weeks or until resolved to Grade 0-1;** If after 2 weeks there is no resolution patient is off study.
Grade 3: blistering, ulceration, or swelling interfering with walking or normal daily activities; cannot wear regular clothing; **Delay dosing up to 2 weeks or until resolved to Grade 0–1.** Decrease dose by 25% and return to original dose interval. If after 2 weeks there is no resolution, PLD should be discontinued.
Grade 4: diffuse or local process causing infectious complications, or a bed ridden state or hospitalization; Off study.

Sample Size Considerations
The anticipated accrual rate is approximately 2 patients per month. The duration of accrual during this phase is a function of the unknown dose-toxicity relationship and the resulting number of dose levels required.

Statistical analysis
Statistics were done with SPSS v15.0 (SSPS, Chicago, IL). Two tailed t-test was used to assess significance in T-cell phenotype data, while non-parametric one-way ANOVA, with Dunn's multiple comparisons post-test was used for cytokine data. Responses and correlation to ELISPOT data were analyzed with Chi2- and Mann-Whitney-U tests. Anti-tumor and anti-JO-1 T-cell induction was calculated with two-tailed Fisher's exact test. Survival data were processed with Kaplan-Meier analysis and Log Rank test.

3. CHEMISTRY, MANUFACTURING, AND CONTROL INFORMATION

3.1 Introduction

The materials for the toxicology study and the clinical trial will be made under cGMP conditions by our contract facility. There will be no major differences in the procedures used to produce the recombinant protein for the toxicology and clinical trial lots.

3.2 Name and Address of Manufacturer

The drug substance and drug product are manufactured by:

Fred Hutchinson Cancer Research Center
Biologics Production Core Laboratory
1100 Fairview Ave. N
Seattle, WA 98109

3.3 Drug Substance

3.3.1 Physicochemical Characterization

JO-1: JO-1 is a 6 × histidinyl-tagged, synthetic protein by fermentation in *E. coli*. JO-1 targets DSG2 in intercellular junctions. JO-1 is a biosynthetic sequence with a dimerization domain, a linker domain and a DSG2 binding sequence derived from adenovirus type 3 (the C-terminal fiber knob domain) (Fig.2A). The molecule forms dimers in solution. The predicted isoelectric point and molecular mass of the monomer are 8.8 and 27.8 kDa, respectively.

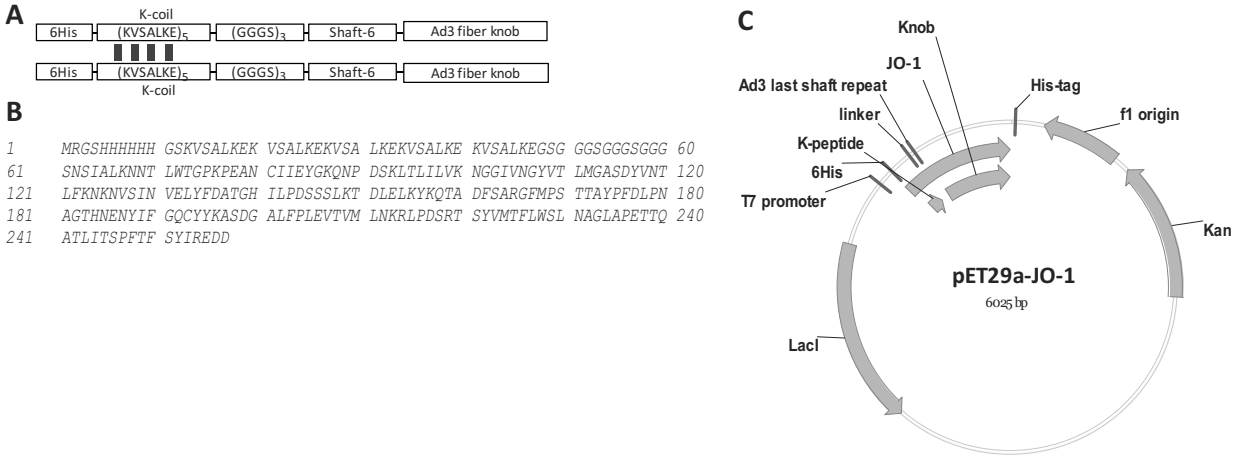


Figure 2: JO-1. **A)** Schematic structure of dimeric fiber knob mutants. The adenovirus serotype 3 fiber knob domain and one fiber shaft motif was fused through a flexible linker to a homodimerizing K-coil domain [3]. The protein is self-dimerizing and can be purified by His-Ni-NTA affinity chromatography. Notably, the fiber knob domain forms a homotrimer. Therefore, JO-1 is a dimer of a trimer. **B)** Predicted amino acid sequence of JO-1. **C)** JO-1 expression plasmid.

PEGylated liposomal doxorubicin (PLD): PLD is a liposomal form of the anthracycline topoisomerase inhibitor doxorubicin. This drug has been in numerous clinical trials, and it is approved for multiple indications including ovarian cancer after failure of platinum-based chemotherapy.

3.3.2 **Method of Preparation of JO-1 Purified Bulk Protein**

3.3.2.1 Raw Materials Used in Production

No materials of primary animal origin will be used in the making of this product; only secondary origin fully traced materials are used. Isopropyl β-D-1-thiogalactopyranoside (IPTG) is made from galactose (milk origin).

3.3.2.2 Expression Construct

JO-1 is produced in *E. coli*. The vector system used is based on the pET system of vectors (Clontech) which utilize a T7 promotor region to drive transcription of the gene of interest. Expression of the T7 RNA polymerase is driven off of a genomic integrant in the expression host and is induced using a lac operon-based system (lacUV5) and an IPTG chemical induction signal. Basal expression of the T7 RNA polymerase, which may cause low levels of protein to be produced prior to induction, is suppressed by expressing T7 lysozyme that enhances growth and yield in the fermenter. The expression plasmid is maintained by the addition of kanamycin to the culture. For manufacturing of JO-1, HMS 174 (DE3) *E. coli* cells carrying the inducible expression plasmid encoding the therapeutic protein are grown overnight to stationary phase and used to seed a large-scale fermentor. After induction, cells are harvested by microfluidization and the supernatant processed to yield the target protein.

3.3.2.3 Master and Working Cell Banks

A Master Cell Bank and a Manufacturer's Working Cell Bank will be manufactured by:

Fred Hutchinson Cancer Research Center
Biologics Production Core Laboratory
1100 Fairview Ave. N
Seattle, WA 98109

All studies on the Master Cell Bank and the Manufacturer's Working Cell Bank will meet the criteria for a valid test and performed in compliance with the requirements of the Food and Drug Administration's Good Manufacturing Practices regulations, 21 CFR Section 211. Studies on the Master Cell Bank include: Induction Assay, Purity in a Bacterial Culture, and Viability Testing. Studies on the Manufacturer's Working Cell Bank were: Induction Assay, Purity in a Bacterial Culture, and Viability Testing. Final Reports will be made available upon request.

3.3.2.4 Fermentation

The fermentations will be performed in a cGMP dedicated 70-L fermenter with a 55-L working volume. Parametric analysis and control will be by Applikon BioExpert software. The inoculant was produced from a working cell bank. A 1:100 dilution is made from a working cell bank into sterile 2 × YS media and then added to a baffled flask containing 2 × YS with kanamycin at a concentration of 5µg/ml. This volume is then incubated in an environmental shaker at 37°C for 16 hours overnight, with agitation set to 240 rpm. The optical density of the inoculant is around 4.5 OD at 600nm at the end of the overnight culture. The fermenter is then inoculated with the overnight culture. The composition of the medium is shown in Table 3.

Table 3: Composition of the 2 × YS Medium used for fermentation.

Reagent	Concentration
Yeast Extract	10 g/L
Soytone, Bacto™	16 g/L
Sodium chloride (NaCl)	5 g/L
Kanamycin sulfate	5 mg/L

Carbon feed reagent is prepared by adding 20 g of monobasic sodium phosphate to a solution of 40% glucose in 2 × YS and adjusting the pH to 7.4. All medium and feed components are sterilized by autoclaving or by sterilizing filtration through a 0.2 µm filter (Carbon feed and Kanamycin).

During the fermentation, the fermenter is maintained at a temperature of 37°C. Air is sparged at a rate of 1 vvm (10 L/min). The pH of the medium was maintained at 7.0 ± 0.2 by automatic addition of acid (5 M H_2SO_4) or base (5 M NH_4OH) as required. The fermenter was programmed to control the dissolved oxygen at 30% by automatically adjusting the agitation, while maintaining a minimum agitation of 500 rpm. Foam control within the fermenter will be achieved by the automatic addition of a silicone antifoam solution. When the cell density reaches a specified level, corresponding to an optical density at 600 nm of approximately 15, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the fermenter to induce expression of the recombinant gene encoding the JO-1 protein. At 2.0 hours post-induction, the fermenter will be cooled and the cells were harvested by ultrafiltration and centrifugation in bottles.

3.3.2.5 Harvest and clarification of bacterial supernatants

Lysis Buffer (50mM NaH_2PO_4, 300 mM NaCl, 10mM imidazole, pH = 8.0) is prepared in 2 liter stocks, sterile filtered, and stored at 2-8 °C. Frozen pellets are placed in ice cold Lysis Buffer at 5 mL per gram wet cell paste and suspended by manual agitation. The pH is re-adjusted to pH = 8.0. Cells are then lysed with four 25 kpsi passages through a cGMP dedicated Microfluidizer (Microfluidics, M-310EH) cooled via internal heat exchanger and circulating chiller (Julabo, SC2500). The debris is clarified by spinning at 4 °C for 1 hour at 33,000 × g.

3.3.2.6 Purification of JO-1

Immobilized metal chelate affinity chromatography (IMAC)

Prior to purification, IMAC-Sepharose 6 Fast Flow resin (GE Healthcare LifeSciences) packed in a BPG column (GE Healthcare LifeSciences) with in-line UV and conductivity monitoring are washed and sanitized with 2M NaOH. The resin is charged with 2 column volumes 0.1 M $NiSO_4$, and equilibrated in Lysis Buffer. Wash Buffer (50mM NaH_2PO_4, 300 mM NaCl, 60 mM imidazole, 20% glycerol at pH 8.0) and Elution Buffer (50mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 20% glycerol at pH 8.0) are prepared, chilled, and attached to the chromatography workstation. The clarified, harvested protein solution is then loaded onto the column. Column is washed with Wash Buffer for 50 column volumes, and bound protein eluted with Elution Buffer until absorbance returned to baseline. Fractions are run on an SDS PAGE gel and fractions with high quality target protein pooled for Anion Exchange Chromatography.

Anion Exchange Chromatography (IEXC)

Phosphate buffered saline (PBS) for elution, Ion Exchange Buffer A (10 mM monobasic sodium phosphate, titrated to pH = 7.2 with NaOH) and Buffer B (50 mM monobasic sodium phosphate, 220 mM NaCl, titrated to pH = 7.2 with NaOH) are prepared. To reduce the ionic strength of the IMAC elution fractions, the pool is diluted 1:5 V:V in Buffer A and loaded column of Q Sepharose Fast Flow (GE Healthcare LifeSciences) equilibrated in Buffer A. Bound material will be washed with 5 column volumes of 17% Buffer B, and JO-1 eluted with PBS while collecting 8 mL fractions; finally residual materials will be eluted and the column washed using 100% Buffer B.

3.3.2.7 In-Process Controls

During the preparation and purification of JO-1 protein, numerous in-process control steps exist to ensure reproducible protein production. Fermentation parameters, including dissolved oxygen, O.D. at 600 nm, agitation, pH, temperature, and consumption of acid, base, and antifoam, are recorded. The yields of both wet cell mass and soluble JO-1 yield are recorded for each fermentation run. SDS-PAGE followed by Coomassie Brilliant Blue Staining is used to follow the JO-1 protein during chromatographic purification. UV and conductivity traces are compared to chromatograms obtained during developmental runs to ensure reproducibility of the chromatographic processes.

SDS-PAGE, size exclusion chromatography and analysis of JO-1 ELISA are used to test the bulk solution for many of the development lots. These assays are not being used as product release test methods.

3.4 Drug Product

3.4.1 Quantitative Statement of Composition

One dose of JO-1 for infusion contains the ingredients listed in Table 4.

Table 4: Composition of Drug Product

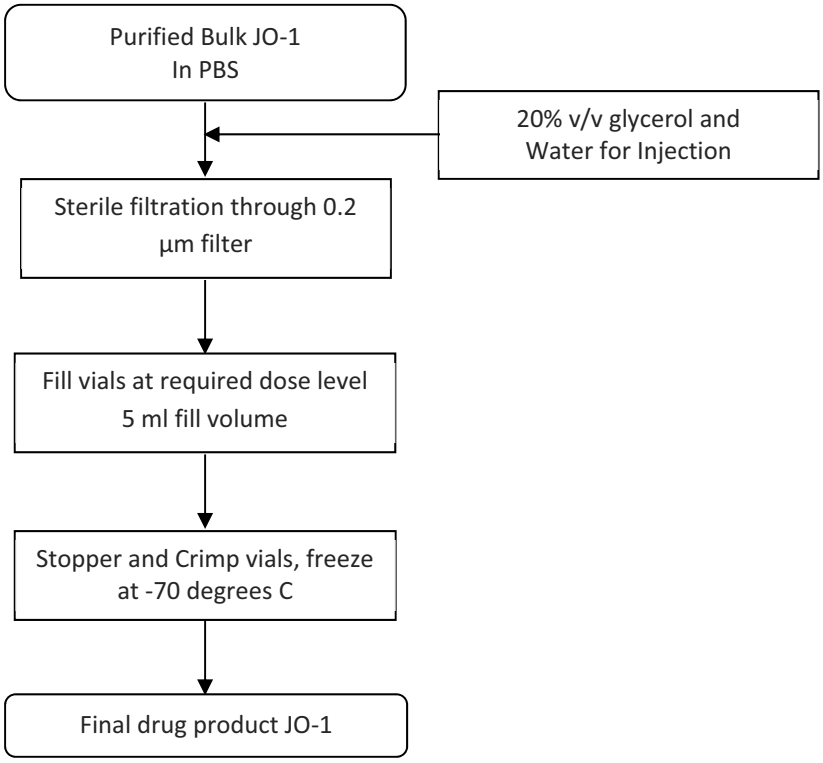
Name of ingredients	Quantity (per kg)	Function	Reference to standards
Active Substances			
JO-1	1 or 4 mg	Junction opening	FHCRC
Excipients			
PBS	90%	pH and osmotic balance	USP
glycerol	10 %	Active protein stability	USP

3.4.2. Potency assay: *PEG permeability assay (see Fig. 7):* A total of $5x10^5$ T84 cells were seeded on 12 mm transwell inserts [PET membrane, with 0.4 m pore size (Corning, NY)] and cultured for 20 days. Culture medium was changed every 2-3 days. TEER was measured. The experiment was started when TEER was constant, i.e. tight junctions had formed. The cells were exposed to JO-1 /JO-4/JO-0 (20 g/ml) in adhesion medium (DMEM, 1% FBS, 2 mM $MgCl_2$, 20 mM HEPES)** for 15 min at room temperature. 1 mCi of [^{14}C] polyethylene glycol-4000 (PEG-4000) (Perkin Elmer, Covina CA) was added to the inner chamber. Medium aliquots will be harvested from the inner and outer chambers and measured by a scintillation counter. Permeability will be calculated as described elsewhere [26]. JO-1 preparations are considered to be functionally potent if they increase PEG permeability >3-fold within 30 min.

3.4.2 **Method of Manufacture of JO-1 for Infusion**

3.4.2.1 *Sterile Filtration*

The JO-1 solution in phosphate buffered saline is mixed with 10% V/V glycerol and is then filter sterilized using a 0.2 µm sterilizing filter. This material is frozen at –20° Celsius and stored as purified bulk.



3.4.3 **Test Methods and Specifications for JO-1 for Infusion**

Testing	**Specifications**
Sterility	No Growth in FTG or SCD
Appearance	Clear, colorless solution
Protein content by AAA	> 0.5 mg/ml
Identity by Western Blot	Positive
Purity by SDS-PAGE (reduced)	> 90% pure as determined by densitometry
Endotoxin content	≤ 15 EU per equivalent maximum human dose
Residual *E. coli* DNA	≤ 100 pg DNA per equivalent maximum human dose
pH	6.5 to 7.5

We will also perform General Safety Tests (see 21 CFR 610.11) on the final vialed product.

3.4.4 **Container / Closure**

The fill volume will be 5 mL. We will use an empty sterilized 5 mL vial (American Pharmaceutical Partners, Inc, SV5, order#6332300105). These are prestoppered and crimped. We will do an aseptic fill using a Cornwall syringe system.

3.4.5 **Stability of JO-1 for Infusion**

While no formal stability testing has been performed on formulated JO-1, the protein demonstrates no loss of activity or noticeable degradation when stored for 9 months at -20 degrees C. An SDS-PAGE gel is shown in Section 4, Figure 3.

3.4.6 **Master Stability Protocol for JO-1**

The Master Stability Protocol for JO-1 is found on the following pages. Stability studies to support storage of the purified bulk at –70°C will be performed as described in 2.

Table 6: Stability Plan for Purified Bulk

Tests	**Purified Bulk (-70°)**							
	Release	*7 days 37°C*	*Month 3*	*Month 6*	*Month 9*	*Month 12*	*Month 18*	*Month 24*
Description	X	X	X	X	X	X	X	X
pH	X	X	X	X	X	X	X	X
Antigen content ELISA	X	X	X	X	X	X	X	X
SDS-PAGE profile	X	X	X	X	X	X	X	X
Western-blot	X	X	X	X	X	X	X	X
Protein content before/ after filtration	X	X	X	X	X	X	X	X
Profile by SEC HPLC	X	X	X	X	X	X	X	X

3.4.7 **Stability tests on the finished product**

Stability studies to support storage of the final container will be performed at +2 to +8 °C as well as after storage of the final container for seven days at 37 °C.

Table 7: Stability plan for final container

Tests	**Final Container**							
	Release	*7 days 37°C*	*Month 3*	*Month 6*	*Month 9*	*Month 12*	*Month 18*	*Month 24*
Description	X	X	X	X	X	X	X	X
pH	X	X	X	X	X	X	X	X
Protein content	X	X	X	X	X	X	X	X
SDS-PAGE profile	X	X	X	X	X	X	X	X
Western-blot	X	X	X	X	X	X	X	X
Potency: Transepithelial permeability	X	X	X	X	X	X	X	X

4. PRECLINICAL STUDIES

4.1 Mechanism of action

4.1.1 **Binding of JO-1 to DSG2**

We identified desmoglein 2 (DSG2) as the main receptor for a group of species B adenoviruses (Ads), including Ad3, a serotype that is widely distributed in the human population. We found that DSG2 interacting domain(s) within Ad3 are formed by several fiber knob domains that need to be in the spatial constellation that is present in whole viral particles. Based on this finding, we generated a small recombinant, self-dimerizing protein containing the Ad3 fiber knob, JO-1. The non-denatured form of JO-1 has a molecular weight of 60kDa as shown by electrophoresis (Fig.3A, "UB"). Boiling of JO-1 in Laemmli buffer disrupts the dimer formed by the K-coil motif as well as the trimer formed by the fiber knob resulting in monomeric Ad3 fiber knobs (Fig.3A "B" lane). Negative stain electron microscopy of purified JO-1 demonstrates the presence of dimers of the trimeric fiber knob (Fig.3B). The atomic structure of JO-1 was delineated by X-ray crystallography (Fig.3C). This analysis clearly shows the three monomers of the trimeric fiber knob. JO-1 efficiently and specifically blocked infection of cells by an Ad3-GFP vector (Ad3) (Fig.3D). Surface Plasmon Resonance analyses showed efficient binding to recombinant DSG2 with an affinity (K_D) of 0.25nM (Fig.3E). These studies show that JO-1 represents the minimal DSG2-binding domains of Ad3.

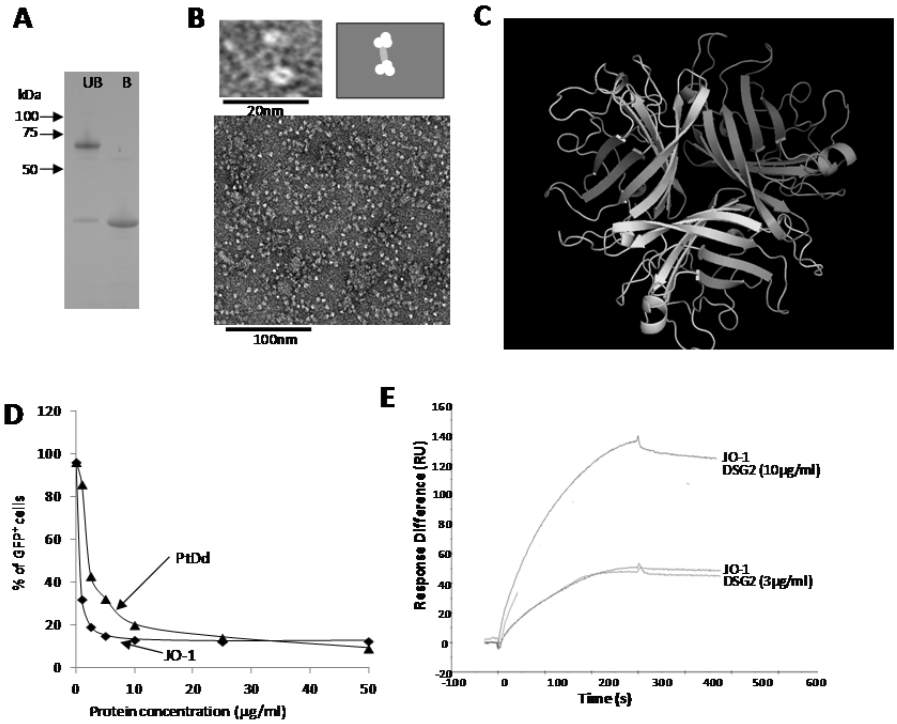


Figure 3. Analysis of JO-1 and its binding to DSG2. A) Coomassie blue stained gel. Samples were run on a 4-15% gradient polyacrylamide gel in Tris/glycine/0.1% SDS buffer. UB-unboiled samples, B-boiled samples. Note that boiling in Laemmli buffer disrupts the trimeric protein structures resulting in fiber knob monomers. **B)** Negative stain electron microscopy of purified JO-1. The upper left image shows fiber knob dimers. Note that the fiber knob itself is a trimer. The right upper panel shows a schematic drawing of the photograph. **C)** Structure of the Ad3 fiber in JO-1. JO-1 was subjected to X-ray crystallography and its 3D structure was resolved at the atomic level. **D)** Competition of Ad3-GFP infection. The vector contains a GFP expression cassette. HeLa cells were incubated with

increasing concentrations of JO-1 or recombinant Ad3 penton-dodecahedra[27] for 60 minutes and then infected with Ad3-GFP at an MOI of 100 pfu/cell for 60 min, after which the viruses were removed and new medium added. GFP fluorescence was measured 18 hours later. N=3. **E)** SPR analysis of JO-1 interaction with DSG2. Biotinylated JO-1 was immobilized to streptavidin-linked sensorchips. DSG2 was injected at indicated concentrations (3 and 10μg/ml). Response signals were collected over the indicated time periods with automatic background subtraction.

We used mutagenesis-based approaches to identify the amino acid residues within the Ad3 fiber knob that are critical for binding to DSG2. The identified residues were in four different areas of the Ad3 fiber knob and formed a potential binding pocket localized in a groove at the top of the fiber knob, facing the receptor (Fig.4).

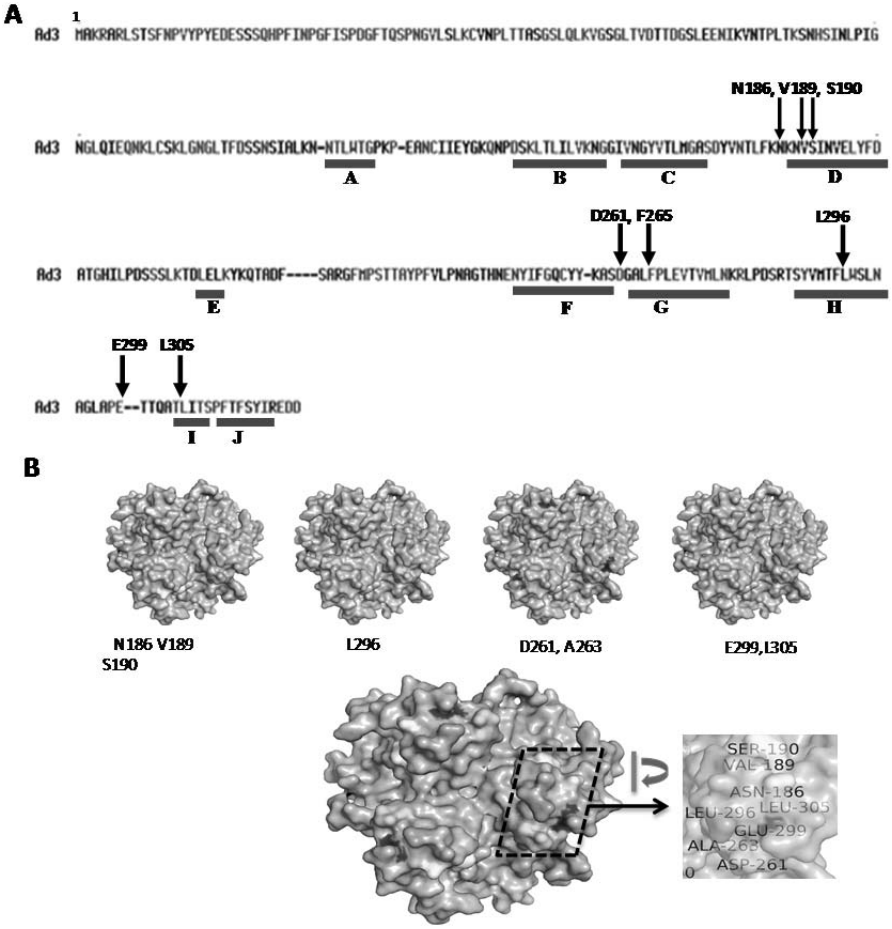
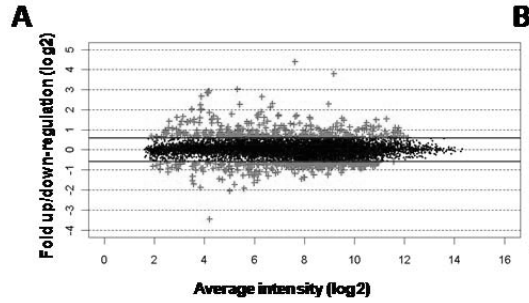


Figure 4. Amino acid residues within the Ad3 fiber knob that are involved in binding to DSG2. A) Shown is the amino acid sequence of the Ad3 fiber knob. Beta sheets present in the Ad3 knob (PDB accession number 1H7Z_A) are indicated by blue lines. Black arrows indicate residues within the Ad3 fiber knob which, when mutated individually, ablate or reduce binding to DSG2. **B)** 3D model of the Ad3 fiber knob. Upper panel: Four critical areas involved in DSG2 binding. The critical residues are shown on the pink isosurface of the trimeric fiber knob. View from the top (apical side) facing the receptor. Lower panel: All critical residues combined. Right side: An enlargement of the groove after a slight side rotation.

4.1.2 **Effect on epithelial cancer cells *in vitro***

4.1.2.1 EMT signaling

Our initial studies were performed with recombinant, purified penton-dodecahedra (PtDd)[27], consisting of 12 Ad3 penton base proteins with protruding fibers. The DSG2 binding domains within PtDd are the same as in JO-1. However, PtDd is a particle with a diameter of 10nm. It has to be produced in insect cells and its purification is not straightforward. BsDd are penton-base dodecahedra without fibers and do not bind to DSG2. To identify intracellular signaling pathways triggered by PtDd interaction with DSG2, we studied mRNA expression profiles (Fig.5A,B). Twelve hours after incubation of polarized BT474 cells with PBS, BsDd, or PtDd, mRNA was analyzed using Affymetrix human ST gene arrays. We found that PtDd treatment resulted in >1.5-fold upregulation of 430 genes and >1.5-fold down-regulation of 352 genes when compared to PBS-treated cells. The list of altered genes was further processed by Pathway-Express software [28]. This computation suggested that PtDd mediated marked activation of a number of signaling pathways involved in EMT, including phosphatidylinositol (PI), mitogen activated protein kinase (MAPK aka ERK), Wnt, adherens junctions, focal adhesion, and regulation of actin cytoskeleton signaling pathways (Fig.5B). Western blot analysis using phosphorylation-specific antibodies showed that PtDd, but not BsDd, triggered the activation of PI3K and MAPK/ERK1/2, i.e. key kinases involved in EMT (Fig.5C). PtDd activation of pathways was mediated by DSG2, because MAPK/ERK1/2 and PI3K phosphorylation was decreased in cells transfected with DSG2 siRNA, but not in control siRNA treated cells. Finally, PtDd-triggered phosphorylation of kinases was absent when cells were pretreated with the ERK1/2 inhibitor UO126 or the PI3K inhibitor Wortmannin. Taken together, our data suggest that PtDd/JO-1 binding to DSG2 triggers EMT in epithelial cells.



B

Deregulated pathways	Number of deregulated genes in pathway	Corrected p-value	Impact factor
Phosphatidylinositol signaling system	3/76	1.20 E-14	35.69
Adherens junctions	4/78	1.43 E-5	13.85
MAPK signaling pathway	5/272	1.60 E-1	3.29
Wnt signaling	5/152	2.79 E-1	2.54
Focal adhesion	5/203	3.02 E-1	2.43
Regulation of actin cytoskeleton	7/217	4.29 E-1	1.91

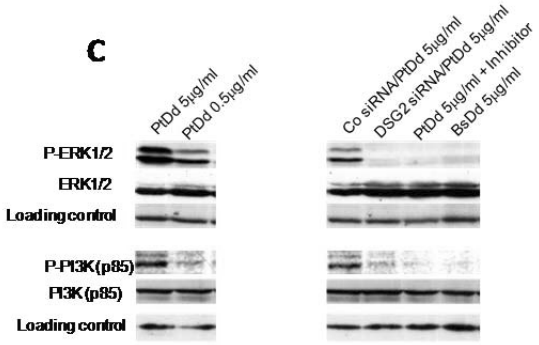


Figure 5. Epithelial-to-mesenchymal transition signaling induced by Ad3 penton-dodecahedra in epithelial cells. A) Graphic demonstration of array data for up- and down-regulated genes (PtDd vs. BsDd treated cells). Each dot represents one gene. **B)** Pathways found to be deregulated at 12 hours after incubation of BT474 cells with PtDd compared to BsDd treatment. Shown are the numbers of deregulated genes in a given

pathway as well as the predicted impact that deregulation of these genes has for the pathway. The impact factor of the entire pathway includes a probabilistic term that takes into consideration the proportion of differentially regulated genes on the pathway and gene perturbation factors of all genes in the pathway. (The gene perturbation factor reflects the relative importance of each gene for the pathway.) A pathway is considered activated when the expression of crucial stimulating genes is upregulated and/or the expression of and inhibitory genes is downregulated. **C)** Western blot analysis of ERK1/2-MAPK and PI3K phosphorylation analyzed 6 hours after incubation of BT474 cells with PtDd or BsDd at the indicated concentrations. For pathway inhibition, cells were treated overnight with Erk1/2 inhibitor UO126 (5 μM) or PI3K inhibitor Wortmannin (2.5 μM) before PtDd was added. The efficacy of the drugs for inhibition of the specific pathway was validated in a previous study [29]. GAPDH is used to demonstrate equal loading.

4.1.2.2 Junction opening

The functional activity of JO-1 was tested in vitro on polarized colon cancer T84 cells. Incubation of T84 cells with JO-1 triggered remodeling of epithelial junctions, as shown by confocal microscopy for claudin 7 and DSG2 (Fig.6). Figures 6A shows confocal immunofluorescence microscopy images of polarized colon carcinoma T84 cells. Shown are the cells from the lateral side, i.e. stacked XZ-layers. In the upper panel, intercellular junctions are visible as long vertical streaks marked by the adhesion junction protein claudin 7. DSG2 (green) is localized at the apical end of claudin 7 signals. The tight junction protein ZO-1 can be found further apical of DSG2 (lower panel). The latter is also visualized in XY images, which show a "chicken-wire" network of tight junctions marked by ZO-1 at the apical cell surface, whereas a section taken 1μm deeper shows DSG2 staining (Fig.6B). Importantly, exposure of T84 cells to JO-1 triggered partial dissolution of epithelial junctions, reflected by decreased staining for DSG2 and ZO-1 (Fig.6C), in comparison to untreated cells (Fig.6A, lower panel). Opening of the tight junctions, which are localized apical to the desmosomal and adherence junctions, is illustrated by electron microscopy (Figs.6D,E). Electron microscopic images of untreated epithelial cells show intact tight and desmosomal junctions as judged by the exclusion of the apically applied dye ruthenium red from basolateral space (Fig.6D, left panel). The dye appears as an electron-dense line along the cell membrane surface. Incubation of epithelial cells with JO-1 resulted in leakage of ruthenium red deep into the lateral space within 1 hour of JO-1 addition (Fig.6D right panel). Disassembly of desmosomes (marked by arrows) in JO-1 treated cells is clearly visible in Figure 6E. Exposure of polarized epithelial cells to JO-1 also increased the transepithelial permeability, as shown by transflux of ^{14}C-PEG-4000 with a molecular weight of 4000 Da (Fig.7A). Importantly, monoclonal antibodies against different regions of the extracellular domain of DSG2 did not significantly increase transepithelial permeability. We speculate that the ligation of several DSG2 molecules is required to trigger the opening of the junctions. Finally, transient opening of junction was confirmed by measuring the transepithelial electrical resistance (TEER) in polarized epithelial cells (Fig.7B). JO-zero is a JO-1 mutant that is abalated for DSG2 binding. Notably, JO-1 had no significant effect on the TEER when studies were done in subconfluent cell cultures where mature junction had not yet formed (i.e. when TEER was not constant). Taken together, exposure of polarized epithelial tumor cells to JO-1 triggers the opening of tight junctions.

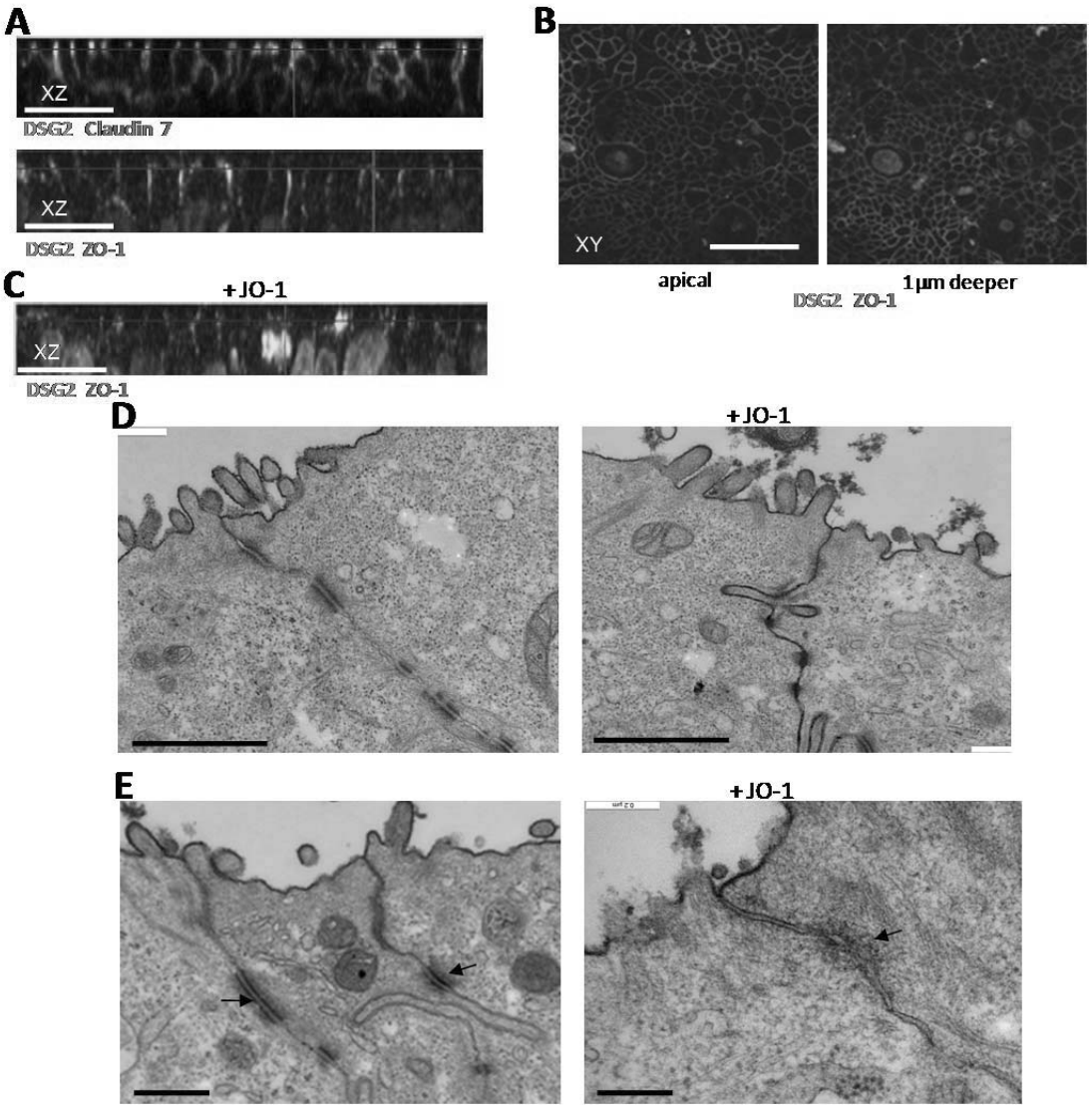


Figure 6. Analysis of epithelial junctions. Studies were performed on polarized colon carcinoma T84 cells cultured for 20 days in transwell chambers. **A)** Confocal immunofluorescence microscopy. Shown are representative stacked XZ images. Upper panel: DSG2 (green) appears at the apical site of baso-lateral junctions marked by claudin 7 (red). Lower panel: The tight junction marker ZO-1 (red) is localized at the apical side of DSG2 (green). Claudin 7 staining masks the lower part of DSG2 "streaks" in the lateral membrane, while ZO-1 staining covers the upper part of DSG2 signals. The scale bar is 20µm. **B)** Shown are XY sections from the cell surface and 1mm deeper stained for DSG2 (green) and ZO-1 (red). **C)** Cells were treated with JO-1 (5µg/ml) and analyzed 12 hours later for DSG2 and ZO-1. **D)** Transmission electron microscopy of junctional areas of T84 cells. Cells were either treated with PBS (left panel) or JO-1 (right panels) for one hour on ice, washed, and then incubated for 1 hour at 37°C. At this time, the electron-dense dye ruthenium red (1) was added together with the fixative. If tight junctions (above the desmosomes) are closed, the dye only stains the apical membrane (black line). If tight junctions are open, the dye penetrates between the cells and stains the baso-lateral membrane. The scale bar is 1µm. Magnification is 40,000x. **E)** A larger magnification (100,000x) shows the disintegration of desmosomes (marked by an arrow) after JO-1 treatment. The scale bar is 0.2 µm.

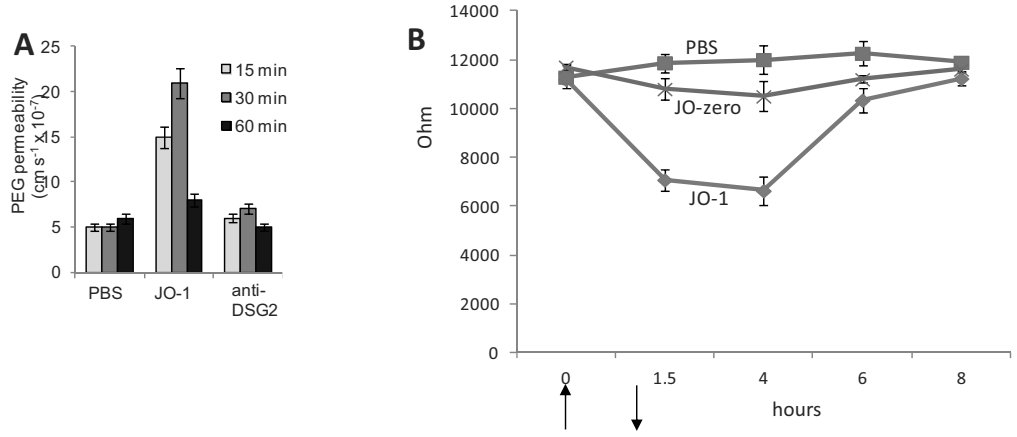


Figure 7. JO-1 increased permeability of junctions. **A)** ^{14}C-PEG-4,000 diffusion through monolayers of T84 cells at different time points after adding JO-1 or anti-DSG2 antibody 6D8 (directed against ECD3/4). **B)** Transepithelial electrical resistance (TEER) measured on polarized colon cancer T84 cells. Cells were cultured in transwell chambers until the TEER was constant, i.e. tight junctions had formed. A total of 5μg of JO-1 or JO-zero in PBS was then added for 1 hour to the apical chamber (indicated by arrows). TEER was measured at the indicated time points. N=6. JO-zero is a JO-1 mutant that is ablated for DSG2 binding.

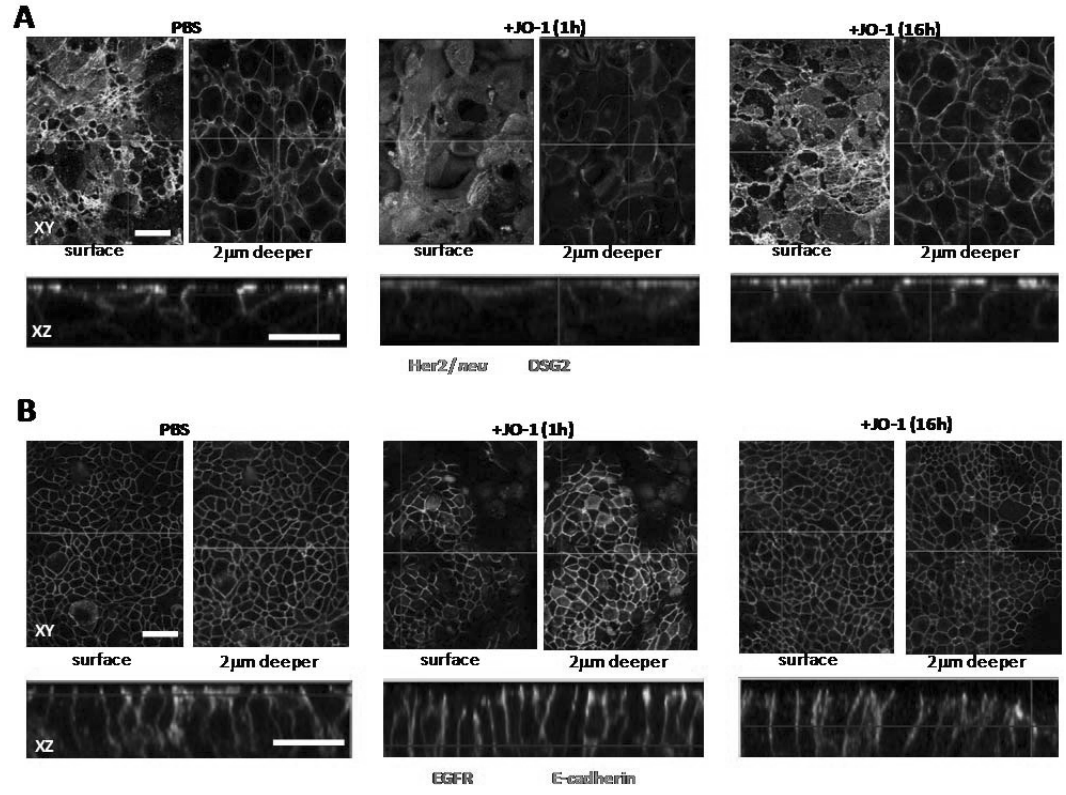


Figure 8. JO-1 induces re-localization of mAb receptors. A) Confocal microscopy of Her2/*neu* (green) and DSG2 (red) staining on polarized BT474 cell cultures (XY and XZ images). Cells treated with PBS are shown in the left panel. Middle and right panels: Cells were treated with JO-1 (20 μg/ml) for 1 h on ice. After removal of JO-1, cells were incubated at 37 °C and analyzed 1 h and 16 h later. XY images show the cell surface (left) and a section 2 μm below the cell surface. The scale bar is 40 μm. **B)** Confocal microscopy of EGFR (red) and the TJ protein E-cadherin (green) on polarized A549 lung cancer cells.

4.1.2.3 Increased access to mAb target receptors

In breast cancer xenograft sections and in cultured breast cancer cells, we found co-staining of Her2/*neu* and the adherens junction protein claudin 7 (Fig.8A). Confocal microscopy of breast cancer BT474 cells confirmed the trapping of Her2/*neu* in lateral junctions. Incubation of the Her2/*neu* positive breast cancer cell line BT474 with JO-1 changed the composition of the lateral epithelial junctions within 1 hour. As a result of this, Her2/*neu* staining at the cell surface became more intense, while it faded in areas distal of the cell surface. This suggests that JO-1-mediated junction opening triggered a translocation of Her2/*neu* from the lateral surface to the apical surface. Being trapped in epithelial junctions also appears for EGFR as co-staining for EGFR and the tight junction protein E-cadherin suggests (Fig.8B). In our studies with cetuximab, we focused on a lung cancer model (A549 cells), as most colon cancer cell lines have mutations in K-ras, which confers resistance to cetuximab [30]. Similar to what we observed for Her2/*neu*, incubation of A549 cells with JO-1 resulted in a translocation of EGFR to the cell surface. JO-1 increases the access to therapy target receptors.

4.1.3 **JO-1 effect on tumors *in vivo***

An orthotopic breast cancer xenograft model (HCC1954) was used to study the effect of JO-1 on epithelial junctions *in vivo*. HCC1954 xenograft tumors resemble the histology of breast cancer in humans [31], i.e. tumors are vascularized and contain nests of epithelial cells "glued" together by epithelial junctions and surrounded by extracellular matrices. JO-1 was injected intravenously into mice with pre-established tumors. JO-1 could be detected in the tumors by immunofluorescence microscopy as early as 1 hour post-injection. JO-1 accumulated in the tumors as is indicated by the increased immunofluorescence at 12 hours post injection (Fig.9A, left three panels). This is also confirmed by Western blot analysis of tumor lysates (Fig.9A, right panel). Analysis of DSG2 on tumor sections by immunofluorescence microscopy in PBS-treated animals showed membrane localized signals (Fig.9B, left panel). One hour subsequent to JO-1 injection, DSG2 molecules were mostly found in the cytoplasm of the tumor cells (second panel). By 12 hours post-injection, membrane localization of DSG2 appeared to be partially restored (third panel). Western blot analysis using anti-DSG2 antibodies against the extracellular domain of DSG2 revealed smaller fragments of the DSG2 (80 and 45kDa) at the 1-hour time point post-injection (Fig.9B, right panel). These fragments represent the extracellular domains (ECD) and proteolytic cleavage products of the ECD. Proteolytic cleavage of DSG2 into stable fragments in normal epithelial tissue and cancer has been reported before [32-34]. In our studies with xenograft tumors, we found less non-phosphorylated and phosphorylated forms of E-cadherin in tumors 12 hours after intravenous injection of JO-1 (Fig.9C, left panel). A transient increase in phosphorylated Erk1/2 preceded the changes in E-cadherin (Fig.9C, compare pErk1/2 PBS vs. JO-1 (1h)). The decrease in E-cadherin and increase in signals for phosphorylated Erk1/2 upon JO-1 injection were also observed by immunofluorescence microscopy (Fig.9C, right panels). These studies indicate that JO-1 triggers transient activation of Erk1/2 pathways *in vivo*. Taken together, intravenous injection of JO-1 resulted in cleavage of DSG2 and activation of EMT pathways within one hour after injection.

We tested whether JO-1-triggered opening of epithelial junctions in tumors would increase the penetration of mAbs into xenograft tumors. The anti-Her2/neu humanized IgG1 mAb, trastuzumab was injected intraperitoneally at a dose of 10 mg/kg into Her2/neu positive HCC1954 tumor-bearing mice [35]. In tumor sections and Western blot analyses, trastuzumab was detectable 1 hour post-injection and at higher levels 12 hours after injection (Figs.10A and B). Quantitative analysis of human IgG1 in tumor lysates by ELISA showed ~6 fold higher levels in mice that received JO-1 injection + trastuzumab (12 h time point) compared to mice that received trastuzumab alone (Fig.10C).

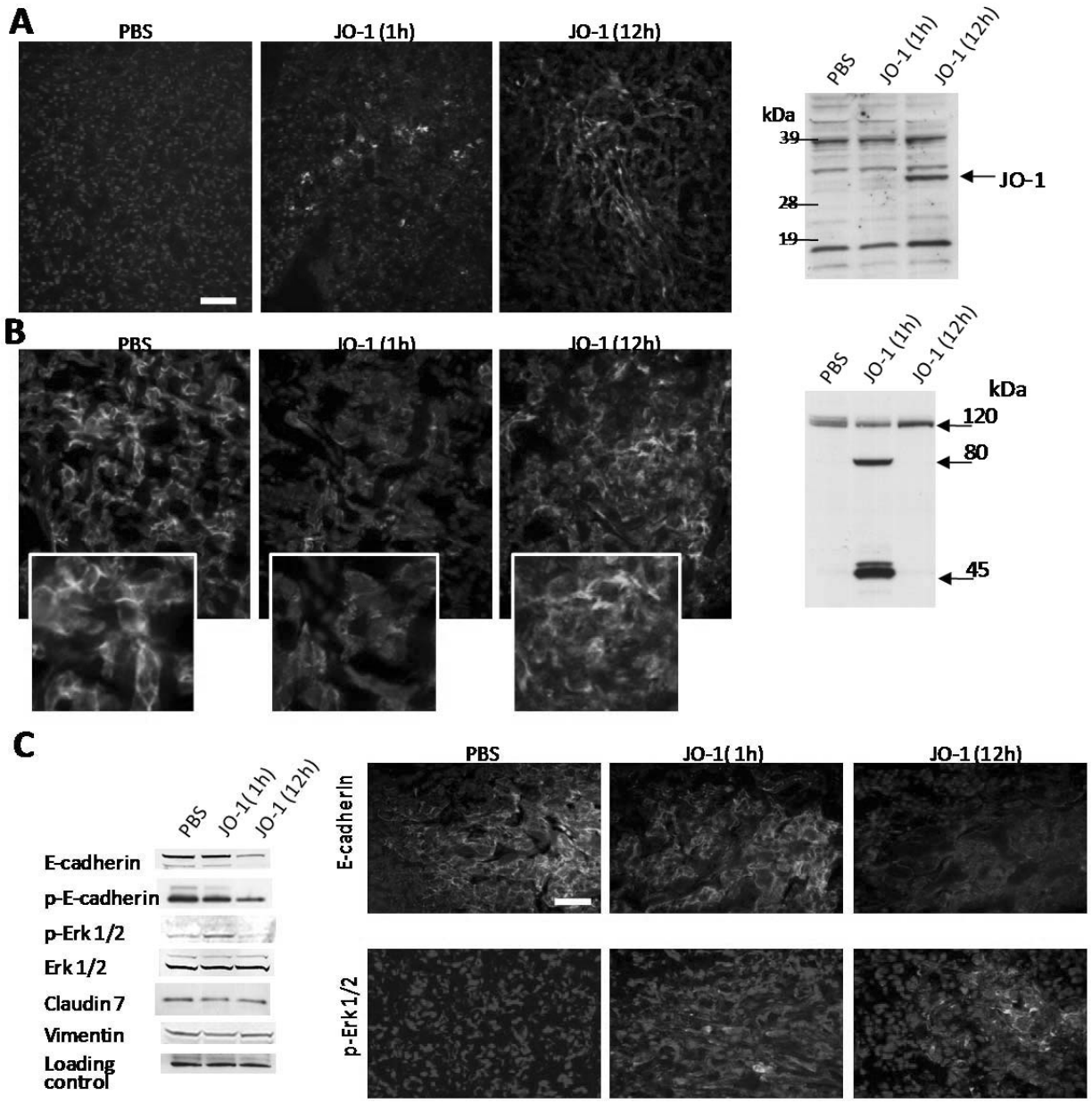


Figure 9. Analysis of mechanism of JO-1 action in tumors in an orthotopic (HCC1954) breast cancer model. When tumors reached a volume of ~200 mm^3, JO-1 (2 mg/kg in 200 μl PBS) was injected intravenously. Tumors were harvested either 1 or 12 h after JO-1 injection. Control mice received 200 μl PBS and tumors were collected 1 h later. **A)** Kinetics of JO-1 accumulation in tumors. Left panels: immunofluorescence analysis of tumor sections using anti-His tag antibodies (for visualization of JO-1). The scale bar is 20 μm. Right panel: Western blot analysis of tumor tissue using Ad3-fiber knob specific antibodies [1]. **B)** Analysis of DSG2 in tumors. Left panel: immunofluorescence analysis of tumor sections using DSG2 antibodies (mAb 6D8 against extracellular domain 3/4 of DSG2). The inserts show a higher magnification. Right panel: The same anti-DSG2 antibody was used for Western blot analysis of tumor tissue. **C)** Intracellular signaling *in vivo*. Left panel: Western blot analysis of tumor tissue for E-cadherin and phosphorylated E-cadherin, Erk 1/2, phosphorylated Erk1/2, claudin 7, and vimentin. Antibodies against γ-tubulin were used to assess sample loading ("loading control"). Right panels: immunofluorescence analysis using antibodies against E-cadherin and phosphorylated Erk1/2.

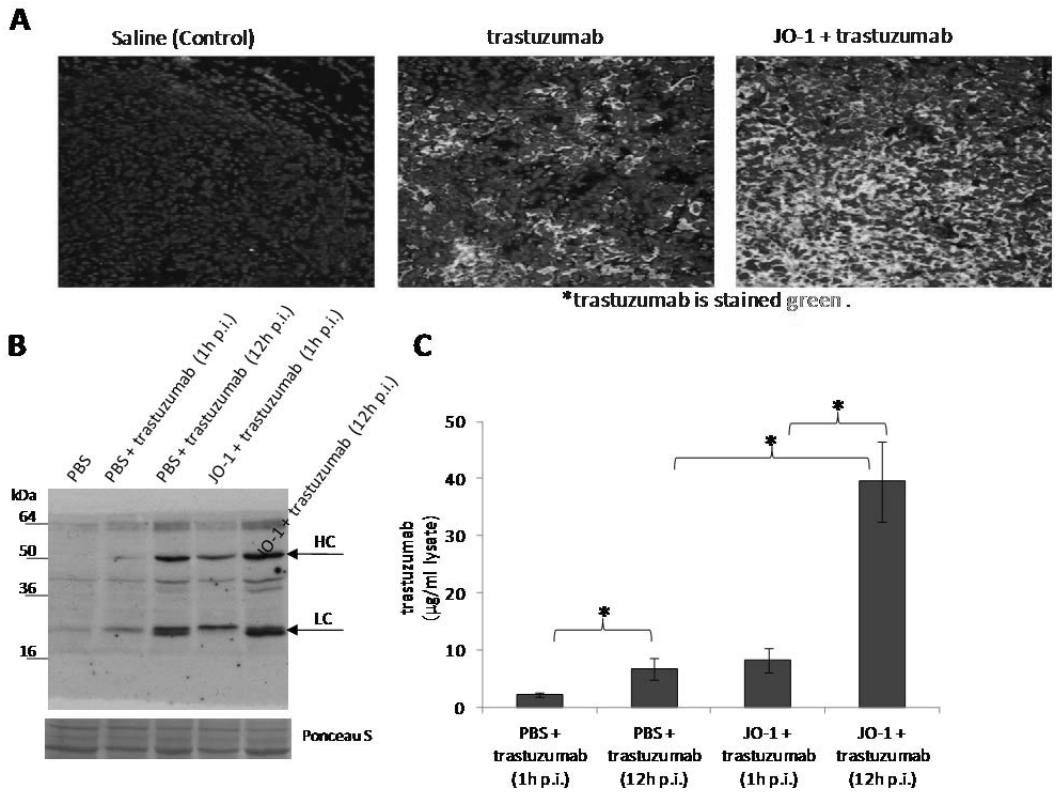


Figure 10. JO-1 improves penetration of trastuzumab in HCC1954 breast cancer tumors *in situ*. Tumor bearing mice were intravenously injected with PBS or JO-1 (2 mg/kg) followed by trastuzumab 1 h later. Tumors were harvested 1 h or 12 h after trastuzumab injection. **A)** Sections (12 hours after injection) were stained for human IgG (i.e. trastuzumab). Positive staining appears green. The scale bar is 20 μm. **B)** Western blot analysis for human IgG (trastuzumab) in tumors. Heavy (HC) and light (LC) Ig chains are indicated by arrows. **C)** ELISA for human IgG1 in tumor lysates. Total protein concentration in all lysates was adjusted to 5 μg/ml. Shown is the ratio of human IgG1 concentrations in tumors of PBS treated mice vs mice that received JO-1 and/or trastuzumab treatment. n=3, * $p<0.05$

Taken together, our data suggest that JO-1 triggers junction opening in epithelial tumors through several, potentially related mechanisms: *i)* cleavage of the DSG2 ECD, and disruption of DSG2 dimers between neighboring cells; *ii)* intracellular signaling that leads to a transient decrease of E-cadherin and potentially other junction proteins; and *iii)* changes in the membrane distribution of Her2/*neu*. These changes mediate better intratumoral accumulation and penetration of a mAb used for cancer treatment.

4.2 Mouse models with human xenograft tumors

4.2.1 Therapy studies of a combination of JO-1 with monoclonal antibodies

Xenograft tumors derived from human breast cancer HCC1954 cells are resistant to trastuzmab at a dose of 2mg/kg (Fig.11A). JO-1 pretreatment significantly improved trastuzumab therapy and stalled tumor growth. Furthermore, cetuximab treatment of mice with pre-established subcutaneous A549 tumors did not result in a significant delay of tumor growth when compared to treatment with PBS (Fig.11B). JO-1 was injected intravenously or intraperitoneally followed by cetuximab 1 hour later. Both treatment approaches had a significant therapeutic effect and resulted in a decrease of tumor volumes.

An additional combination of intravenously injected JO-1 with an intratumoral application of the junction opener did not further increase the therapeutic efficacy. As seen in the breast cancer model, JO-1 treatment alone did not exert a significant anti-tumor effect.

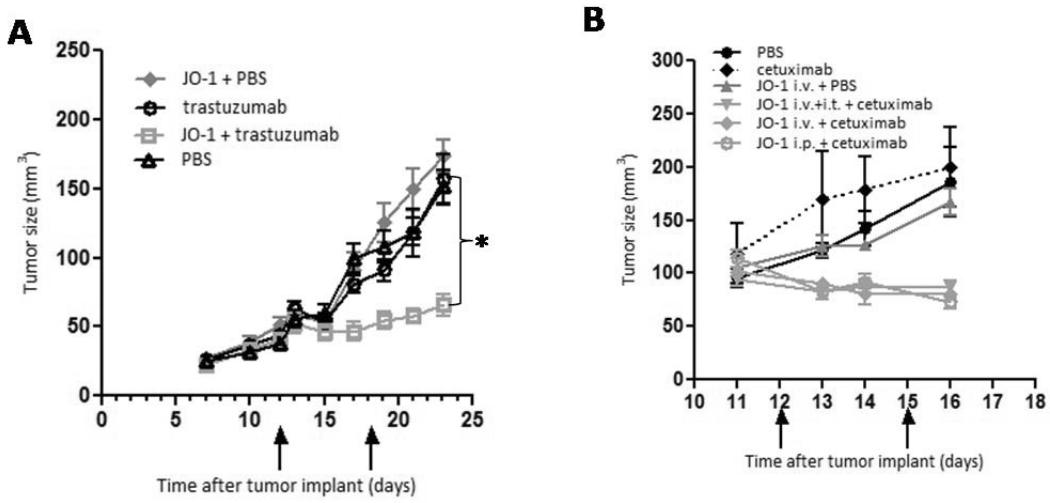


Figure 11. JO-1 + mAbs halt tumor growth. A) JO-1 improves trastuzumab therapy in Her2/*neu* positive breast cancer model. BT474-M1 breast cancer model: When tumors reached a volume of ~100 mm^3, mice received an intravenous injection of JO-1 or PBS, followed by an intraperitoneal injection of trastuzumab (2mg/kg) or PBS 1 h later. Arrows indicate treatment cycles. **B)** JO-1 improves cetuximab therapy in lung cancer xenograft models. Subcutaneous A549 tumor model: JO-1 was injected at day 12 and 15 intravenously (2 mg/kg) or intraperitoneally (4 mg/kg) followed by an intraperitoneal injection of cetuximab or PBS 1 h later. One group received 1 mg/kg of JO-1 intravenously and 1 mg/kg intratumorally. n = 5. All JO-1 cotherapies are significantly more effective than trastuzumab alone. The difference between JO-1 injection routes was not significant.

The co-therapy approach was tested in a metastatic lung cancer model. In this model, mice became morbid within 37 days of tumor cell transplantation with predominant tumor localization to the lung (Fig.12 "PBS" group). Treatment of mice with JO-1/cetuximab was started at day 10. All animals were sacrificed at day 40. While lung metastases were clearly visible in the control group, JO-1 group, as well as the cetuximab treated animals, 80% of the lungs in the JO-1+cetuximab-treated animals were free of tumor when inspected macroscopically. Microscopy of lung sections showed that in PBS treated-animals, tumor cells almost completely replaced normal lung tissue and also filled the bronchioles. (Fig.12, right panels). While cetuximab treated animals had considerable, infiltrating tumor growth, the majority of JO-1+ cetuximab injected animals showed only micrometastases.

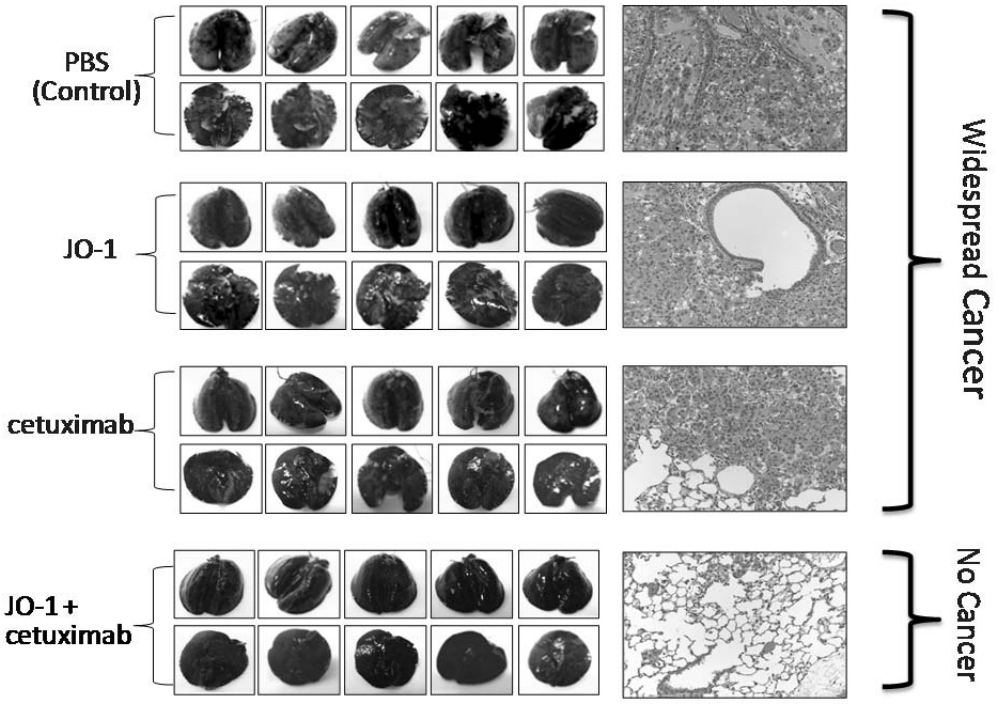


Figure 12. JO-1 improves cetuximab therapy in a metastatic lung cancer models. Metastatic A549 lung cancer model: 10 days after intravenous injection of A549 cells, mice received an intravenous injection of 2 mg/kg JO-1 or PBS, followed by an intraperitoneal injection of cetuximab (10 mg/kg) or PBS 1 h later. The treatment was repeated every 7 days until day 38. n=10. Left panels: Lungs from individual mice stained with India ink. Healthy tissue appears black. Tumor tissue stains white. Right panel: representative sections of lungs stained with H&E.

These studies show that i.v. JO-1 injection one hour before mAb injection can overcome resistance to treatment with trastuzumab and cetuximab.

4.2.2 **Therapy studies of a combination of JO-1 with chemotherapy drugs**

We conducted studies with JO-1 and common chemotherapy drugs with molecular weights >500Da.

4.2.2.1 Studies with nab-paclitaxel, irinotecan, paclitaxel

Co-therapy of JO-1 and paclitaxel was first tested in the BT474-M1 breast cancer model (Fig.13A). These tumors are resistant to paclitaxel. JO-1 co-therapy was able to halt tumor growth and thus overcame resistance to paclitaxel treatment. JO-1 also significantly increased the therapeutic efficacy of irinotecan (Fig.13B).

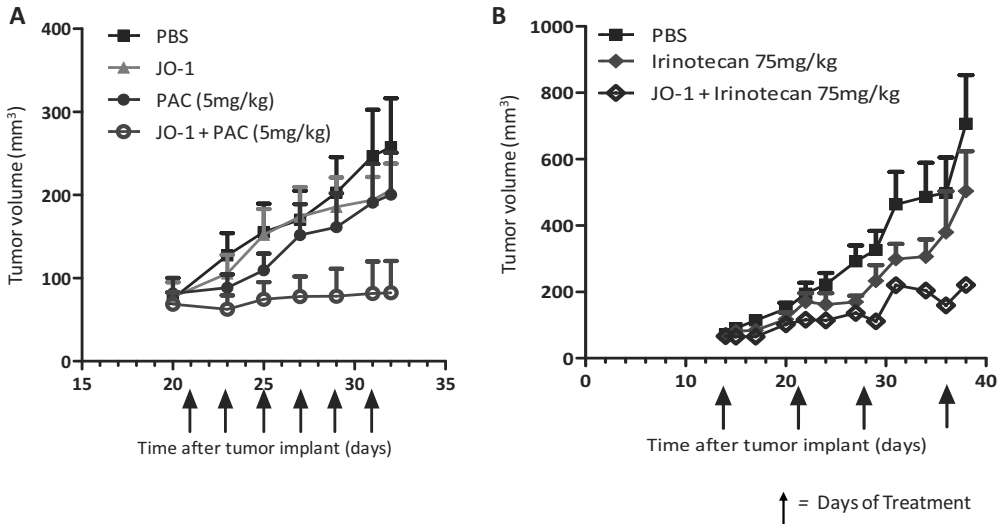


Figure 13. JO-1 + chemotherapy with paclitaxel and irinotecan delays tumor. A) Paclitaxel. A total of $4x10^6$ breast cancer BT474-M1 cells, were injected into the mammary fat pad of CB17-SCID/beige mice. Twenty-one days later when tumors reached a volume of ~80 mm^3, mice received an intravenous injection of 50 µg of JO-1 (2 mg/kg) or PBS, followed by an intravenous injection of paclitaxel (PAC) (5 mg/kg) or PBS one hour later. The treatment was given every other day until day 31. Animals received a total of 6 doses of co-therapy (marked by arrows). n=5. **p<0.05 **B) Irinotecan:** Immunodeficient CB17-SCID/beige mice were injected subcutaneously with $4x10^6$ lung cancer A549 cells. JO-1 (2 mg/kg) was injected at day 14 intravenously (when tumors had a volume of 65 mm^3) followed by an intraperitoneal injection of irinotecan (I) (37.5 mg/kg) or PBS 1 h later. The treatment was repeated weekly until day 36. Shown are tumor volumes. n = 5. irinotecan (37.5 mg/kg*) vs.* JO-1+irinotexan (37.5 mg/kg): P<0.01 on day 38

We furthermore conducted studies in a model for triple-negative breast cancer (TNBC). TNBC is characterized by a lack or minimal expression of estrogen receptor (ER), progesterone receptor (PR) and the absence of Her2/neu overexpression. TNBC accounts for 15% of all breast cancers. Overall survival is poor compared with that in patients who have other phenotypes. Characteristic feature of TNBC are high levels of DSG2 and epithelial junctions. Promising clinical results in the treatment of TNBS have been achieved with nanoparticle, albumin-conjugated paclitaxel (nab-paclitaxel/Abraxane™) alone or in combination with the EGFR-targeting mAb cetuximab/Erbitux™ [36]. Our studies demonstrate that JO-1 significantly increased nab-paclitaxel/cetuximab therapy in a mouse model with orthotopic TNBC tumors (Fig.14).

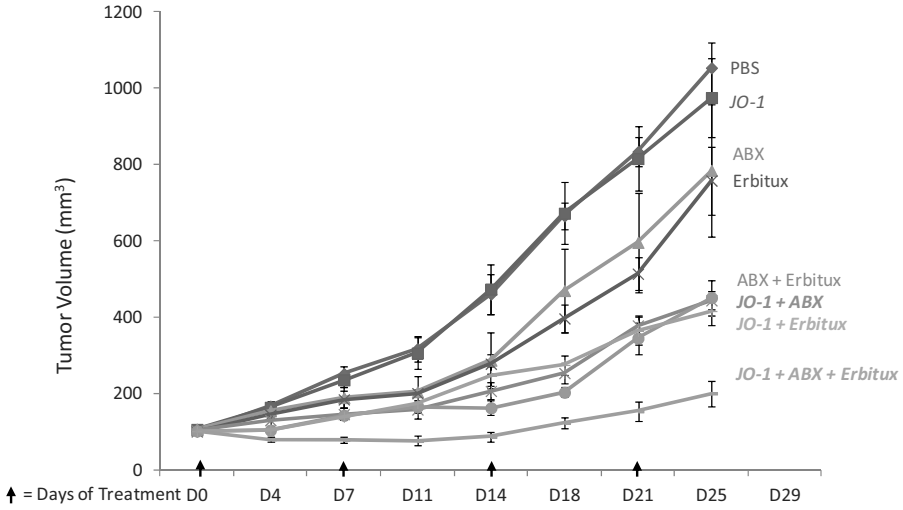


Figure 14. JO-1 enhances therapy in poor-prognosis triple negative breast cancer (TNBC). A total of $4x10^6$ TNBC MDA-MB-231 cells were injected into the mammary fat pad of CB17 SCID-beige mice. JO-1 (2mg/kg) was intravenously injected 1 hour before the application of cetuximab/Erbitux (10mg/kg, i.p.) and nab-paclitaxel/Abraxane (5mg/kg, i.v). Treatment was started on day 0 and then given weekly. N=10 P<0.01 at day 25 for Abraxane vs JO-1+Abraxane, Erbitux vs JO-1+Erbitux, and Abraxane/Erbitux vs JO-1+Abraxane/Erbitux.

Another important finding was that JO-1 allowed for a reduction of the effective doses of chemotherapeutic drugs, for example nab-paclitaxel. Nab-paclitaxel was administered at two different dose levels (5 and 10 mg/kg) alone or in combination with JO-1 (Fig.15). The combination of JO-1 plus nab-paclitaxel (5 mg/kg) was significantly more effective than nab-paclitaxel alone given at a dose of 10 mg/kg. JO-1 plus nab-paclitaxel (10 mg/kg) halted tumor growth.

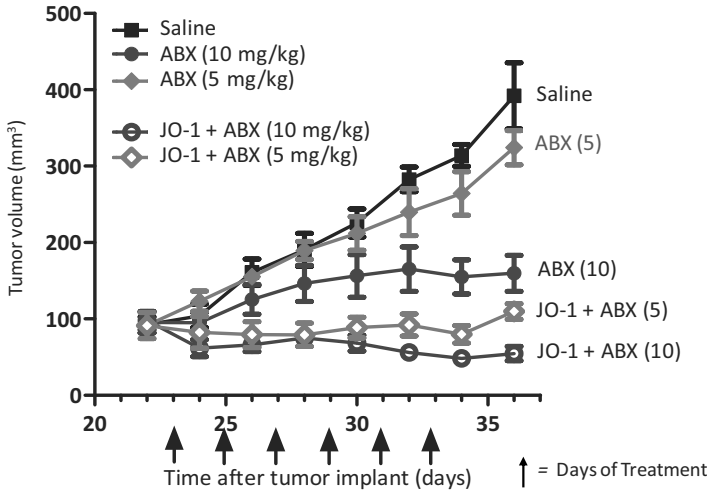


Figure 15. JO-1 enables reduction of nab-paclitaxel dose. Immunodeficient CB17-SCID/beige mice were subcutaneously injected with $3x10^6$ MDA-MB-231 cells. Mice were treated from day 23 on, when the tumors reached a volume of ~ 90 mm^3. They were injected every other day with JO-1 (2 mg/kg) or PBS intravenously, followed 1h later by nab-paclitaxel (ABX) at different dose levels (10 and 5 mg/kg). Shown are tumor volumes. n = 5. ABX (5 mg/kg) *vs* JO-1 + ABX (5 mg/kg): P<0.001 from day 28 on; ABX (10 mg/kg) *vs.* JO-1 + ABX (10 mg/kg): P<0.01 from Day 30 on.

In addition to enhancing the efficacy of anti-cancer drugs, JO-1 also decreased toxic side effects associated with these drugs. Nab-paclitaxel (10 mg/kg) treatment decreased the white blood cell count, which was prevented by JO-1 pre-treatment (Fig.16A). The myeloprotective effect was also shown in sternum sections. In contrast to the depletion of bone marrow cells seen in mice treated with nab-paclitaxel (Abraxane/ABX) alone, animals that received JO-1/ABX co-therapy displayed a normal histology (Fig.16B). Notably, no toxic side effects of nab-paclitaxel were found in the other tissues analyzed, i.e. intestine, kidney and spleen.

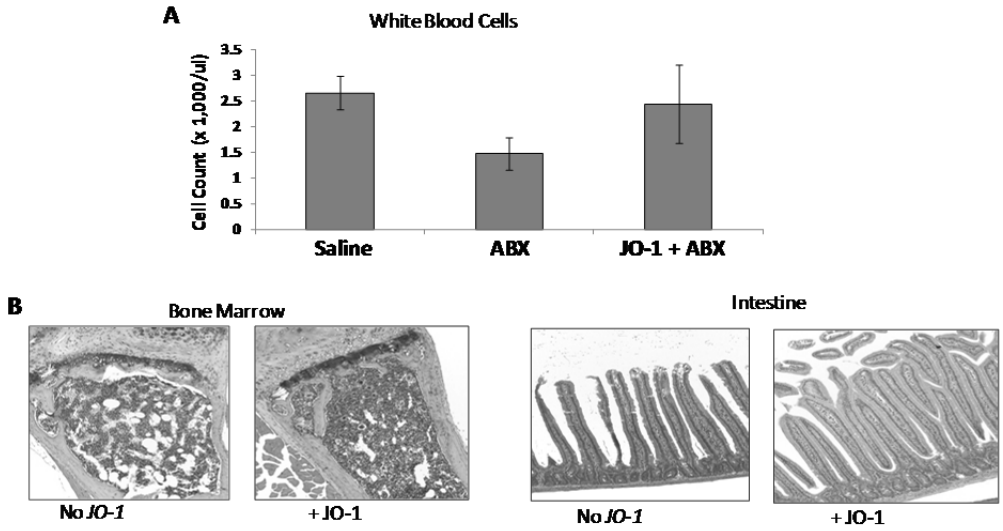


Figure 16. JO-1 prevents toxicities of nab-paclitaxel. Immunodeficient CB17-SCID/beige mice were subcutaneously injected with $3x10^6$ MDA-MB-231 cells. Mice were treated from day 23 on, when the tumors reached a volume of ~ 90 mm^3. They were injected every other day with JO-1 (2 mg/kg) or PBS intravenously, followed 1h later by nab-paclitaxel (ABX) at 10mg/kg. **A)** White blood cell counts in treated mice at the day of sacrifice (day 36). n=5, ABX *vs* JO-1+ABX: *p<0.05. **B)** Representative sternum sections. H&E stained. Note depletion of bone marrow cells in ABX treated mice.

JO-1 also reduced toxicites associated with irinotecan. Irinotecan treatment caused thrombocytopenia and leukopenia as a result of myelotoxicity, although there was no significant difference in the severity of leukopenia or thrombocytopenia observed in animals treated at the the two different doses (Fig.17A). Co-administration of JO-1 had a myelo-protective effect. It prevented the decrease in platelet and white blood cell counts, which was especially noteworthy at the higher drug dose. Compared to control animals, spleens were significantly smaller in irinotecan treated animals but not in animals that received irinotecan in combination with JO-1. Splenic atrophy in irinotecan treated animals is also visible in microscopic analysis of spleen sections (Fig.17B). JO-1 also reduced irinotecan-mediated damage to the intestinal epithelium (Fig.17C).
As discussed below (4.5.), by acting on epithelial junctions of tumors, JO-1 appears to create as a “sink” for therapeutic drugs in tumors, which decreases the levels and exposure of these drugs in normal tissues.

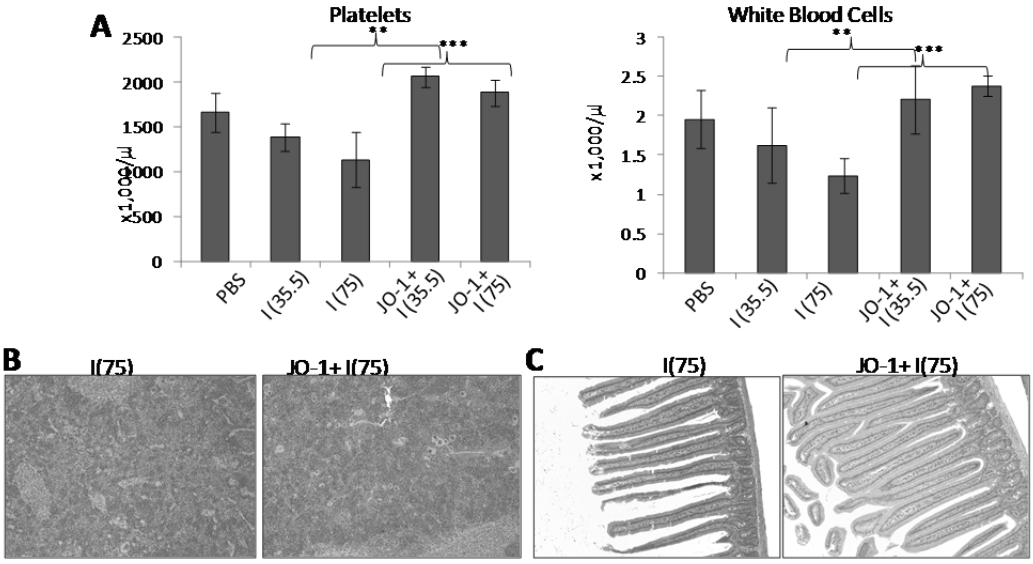


Figure 17. JO-1 prevents toxicities of irinotecan: Immunodeficient CB17-SCID/beige mice were injected subcutaneously with $4x10^6$ A549 cells. JO-1 (2 mg/kg) was injected at day 14 intravenously (when tumors had a volume of 65 mm^3) followed by an intraperitoneal injection of irinotecan (I) at two different dose levels (75 mg/kg or 37.5 mg/kg) or PBS 1 h later. The treatment was repeated weekly until day 36. **A)** Platelet and white blood cell counts in treated mice at the day of sacrifice (day 38). n=5, irinotecan (37.5 mg/kg) *vs.* JO-1+irinotexan (37.5 mg/kg): **P<0.01. irinotecan (75 mg/kg) *vs.* JO-1+irinotexan (75 mg/kg): ***P<0.001. **B)** Representative spleen sections, H&E stained. Note depletion of cells in germinal centers in I(75) treated mice. Magnification: 20x. **C)** Representative intestine sections.

4.2.2.2 Studies with PLD in ovarian cancer models.

Studies were performed in xenograft tumors derived from ovc316 cells [29,37]. Ovc316 cells are Her2/*neu* positive epithelial tumor cells derived from an ovarian cancer biopsy. These cells can undergo EMT and the reverse process, mesenchymal-to-epithelial-transition (MET), under specific conditions *in vitro* and *in vivo*. A subfraction of ovc316 cells that is positive for Nanog, CD133, and E-cadherin is enriched for cancer stem cells, i.e. self-renewing cells with pluripotent potential and tumor forming ability [37]. Ovc316 cells therefore closely model the heterogeneity and plasticity seen in tumors *in situ*. In a first model, ovc316 cells were implanted into the mammary fat pad of immunodeficient mice. The enhancing effect of JO-1 on PLD therapy was shown in two models, in a model with ovc316-derived mammary fat pad tumors (Fig.18A) and in a model with ovc316-derived intraperitoneal tumors (Fig.18B). The latter model reflects the localization of ovarian cancer in patients. In both models, JO-1 and PLD were given intravenously

PLD caused hepato- and myelo-toxicities. As seen with other chemotherapeutic drugs, JO-1 also decreased PLD related toxicities (Fig.19A-C). We speculate that the ability of JO-1 to open up intercellular junctions in tumors and increase the uptake of chemotherapeutics reduces the drug exposure to normal tissue thus providing a larger therapeutic window. In support of this speculation, immunofluorescence analysis of tissue sections revealed more PLD signals in tumors of JO-1+PLD treated mice (Fig.19D). In these animals, PLD was found more dispersed over a greater distance from blood vessels, suggesting better intratumoral penetration and absorption by tumor tissue.

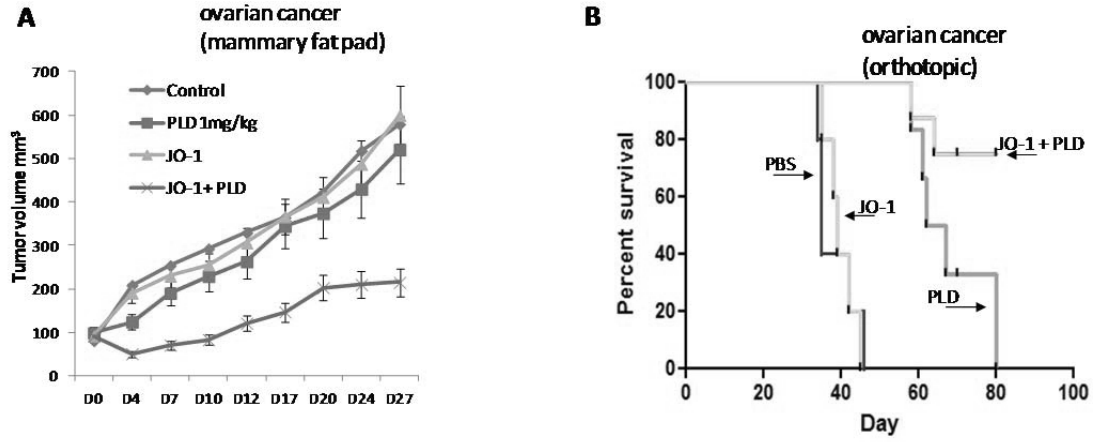


Figure 18. JO-1 enhances PLD therapy in an ovarian cancer model. A) mammary fat pad tumors. Treatment was started when tumors reached a volume of 100mm^3. Mice were injected intravenously with 2 mg/kg JO-1 or PBS, followed by an intravenous injection of PLD (1 mg/kg) or PBS one hour later. Treatment was repeated weekly. **B)** intraperitoneal tumors: CB17-SCID/beige mice with intraperitoneal tumors derived from primary human ovarian cancer cells ovc316. Treatment was started at day 35 after tumor cell implantation and repeated weekly. Mice were injected intravenously with 2 mg/kg JO-1 or PBS, followed by an intravenous injection of PLD (2 mg/kg) or PBS one hour later. Onset of ascites was taken as an endpoint in therapy studies. Shown is the survival of animals in a Kaplan Meier graph. n=10. p <0.001 for PLD vs JO-1 + PLD.

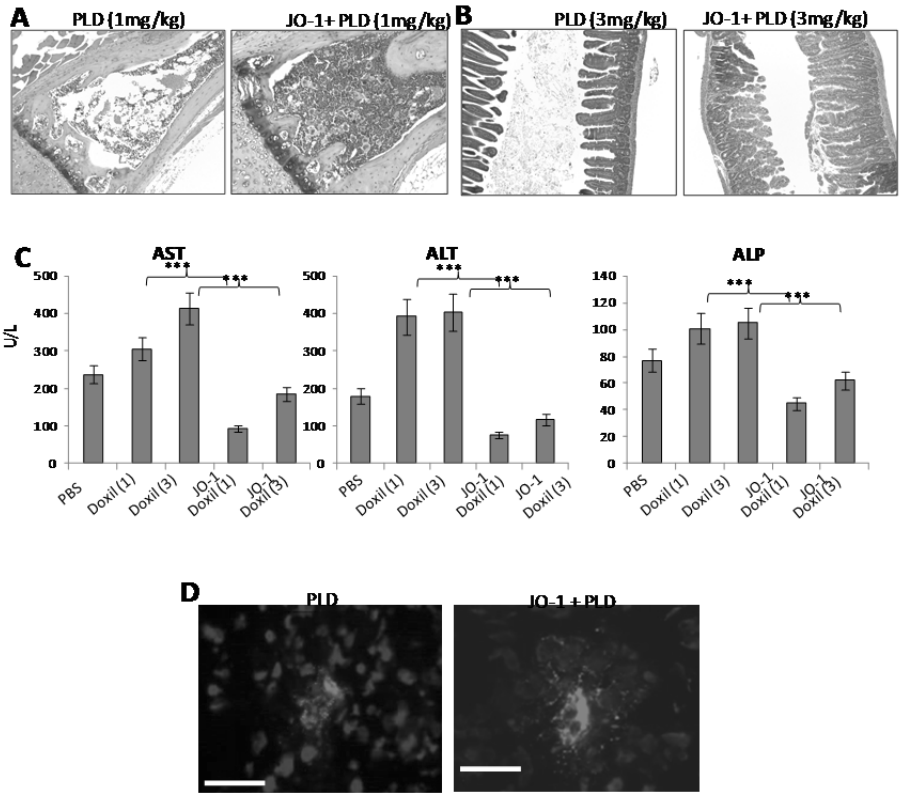


Figure 19. JO-1 allows for less side effects of PLD therapy. Mice with xenograft tumors were injected intravenously with 2 mg/kg JO-1 or PBS, followed by an intravenous injection of PLD (1 or 3 mg/kg) or PBS one

hour later. The treatment was repeated 3 days later **A)** Representative sections of sternum 2 days after the last JO-1/PLD injection. Note that bone marrow is depleted in PLD treated animals. **B)** Representative sections of intestine. **C)** Serum enzymes: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). n=3. PLD (1 mg/kg) vs JO-1+PLD (1 mg/kg): **P<0.01; PLD (3 mg/kg) vs JO-1+PLD (3 mg/kg): **P<0.01. The elevated transaminase levels in PBS- treated control animals may be due to the relatively large size of xenograft tumors in these mice, which can produce factors that damage hepatocytes. **D) PLD penetration in tumors** Mice were intravenously injected with PBS or JO-1 (2 mg/kg) followed by PLD or PBS one hour later. Two hours after PBS or PLD injection, mice were sacrificed and tumor harvested. Immunfluorescence analysis of tumor sections with anti-PEG antibodies. PLT appears in red. The scale bar is 20□m. Notably, free PEG is poorly detected by ELISA or immunohistochemistry [38].

Taken together, we have shown in over 25 xenograft tumor models that the intravenous injection of JO-1 increased the efficacy of monoclonal and chemotherapy, subsequently reducing the required treatment dose and concomitantly reducing the toxic side effect of these treatments.

4.2.3 **Safety studies with JO-1: Risk of metastasis**

JO-1 binding to DSG2 on tumor cells triggers pathways involved in EMT, a process which, as mentioned above, has been associated with tumor metastasis. Over 20 *in vivo* studies conducted with JO-1 combined with a range of cancer therapeutics in various different cancers with long-term follow-up, has not provided any evidence of metastases [23]. Furthermore, at day 3 after JO-1 injection into mice bearing Her2/neu-positive HCC1954 tumors, there was no significant increase in the percentage of circulating Her2/neu- positive cells in the blood (Fig.20). Tumor metastasis requires more than transient activation of EMT pathways. Detachment from epithelial cancers and migration of tumor cells is only possible after long-term crosstalk between malignant cells and the tumor microenvironment, resulting in changes in the tumor stroma and phenotypic reprogramming of epithelial cells into mesenchymal cells [39]. These studies show that JO-1 did not trigger metastasis.

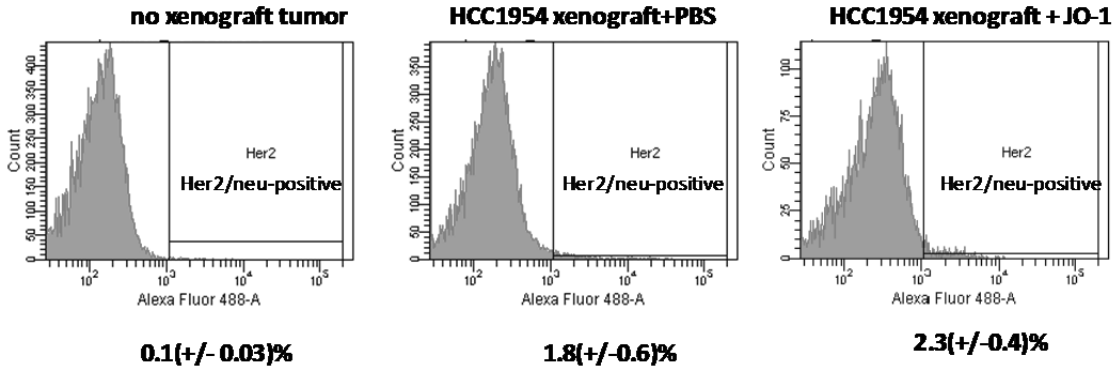


Figure 20. Circulating tumor cells. A total of 4x10^6 Her2/neu-positive HCC1954 cells, were injected into the mammary fat pad of CB17-SCID/beige mice. When tumors reached a volume of ~300 mm^3, mice received an intravenous injection of 50μg of JO-1 (2 mg/kg) or PBS. Twenty-four hours later blood was collected, red cells were lysed and the remaining cells were subjected to flow cytometry with antibodies specific to Her2/neu. n=3. The difference between PBS and JO-1 injected mice is not significant (p=0.16).

4.3 JO-1 reduces hypoxia in tumors

Hypoxia or oxygen deprivation is a key factor in tumor progression and resistance to therapy due to its effect on various metabolic, molecular-genetic, pathophysiologic adaptive processes including neoangiogenesis and activation of immunosuppressive T cells. The most important regulatory factor of the hypoxia-signaling pathway activity in cells is hypoxia-inducible transcription factor 1 (HIF-1).

We have found that intravenous injection of JO-1 into mice with tumors >600mm^3 decreases intratumoral HIF-1-dependent transgene expression. We generated cancer cells that expressed luciferase under the control of a HIF-1-responsive promoter. This was achieved by transducing lung cancer (A549) cells and primary ovarian cancer (ovc316) cells with a hypoxia/luciferase reporter lentivirus vector from SabioSciences (http://www.sabiosciences.com/reporterlenti.php). Clones of transduced cells were analyzed for luciferase expression with and without chemically ($CoCl_2$) induced hypoxia. Two clones with the highest induction factor were selected for in vivo studies. Mice with A549 tumors were subjected to non-invasive in vivo imaging for luciferase expression. With tumor growth luciferase signals (normalized to tumor volume) increased, suggesting hypoxia. When tumors reached a volume of 600mm^3, mice were intravenously injected with JO-1. Images were taken before JO-1 injection (0 hours) and 1, 3, and 6 hours after JO-1 injection (Fig.21). While JO-1 significantly decreased normalized luciferase signals a JO-1 mutant (JO-0) that was ablated for DSG2 binding had no effect. Furthermore, there was no change in luciferase signals upon JO-1 injection into mice with tumors that expressed luciferase under a promoter that was not controlled by HIF-1. Future studies will include measuring intratumoral oxygen concentrations using polarographic oxygen electrodes [40].

This finding that JO-1 decreases hypoxia is relevant for cancer radiotherapy which is often inefficient in hypoxic environments [22]. It will also give us a means to monitor the effect of JO-1 in cancer patients by noninvasive imaging. Hypoxia induces a shift from aerobic to anaerobic glycolysis (Embden-Meyerhof pathway), which is associated with increased glucose utilization in malignant cells. Increased glycolysis reflects increased glucose transport and hexokinase activity, and this increase in glucose use can be imaged using a radiolabeled analogue of glucose [18F]2-fluorodeoxyglucose ([18F]FDG) and PET. Noninvasive whole-body [18F]FDG PET imaging is routinely and widely used in the clinic for tumor diagnosis and staging of the extent of disease [41], as well as for monitoring the efficacy of anticancer therapies [42]. JO-1 reduces tumor hypoxia, which allows us to study the effect of JO-1 on the tumor in cancer patients. At this time, PET imaging is however not yet approved for ovarian cancer. We will therefore use indirect means to assess hypoxia in tumors via serial measurements of serum markers (VEGF) and tissue markers (HIF-1, GLUT-1 and CAIX) in pre and post treatment biopsies when available.

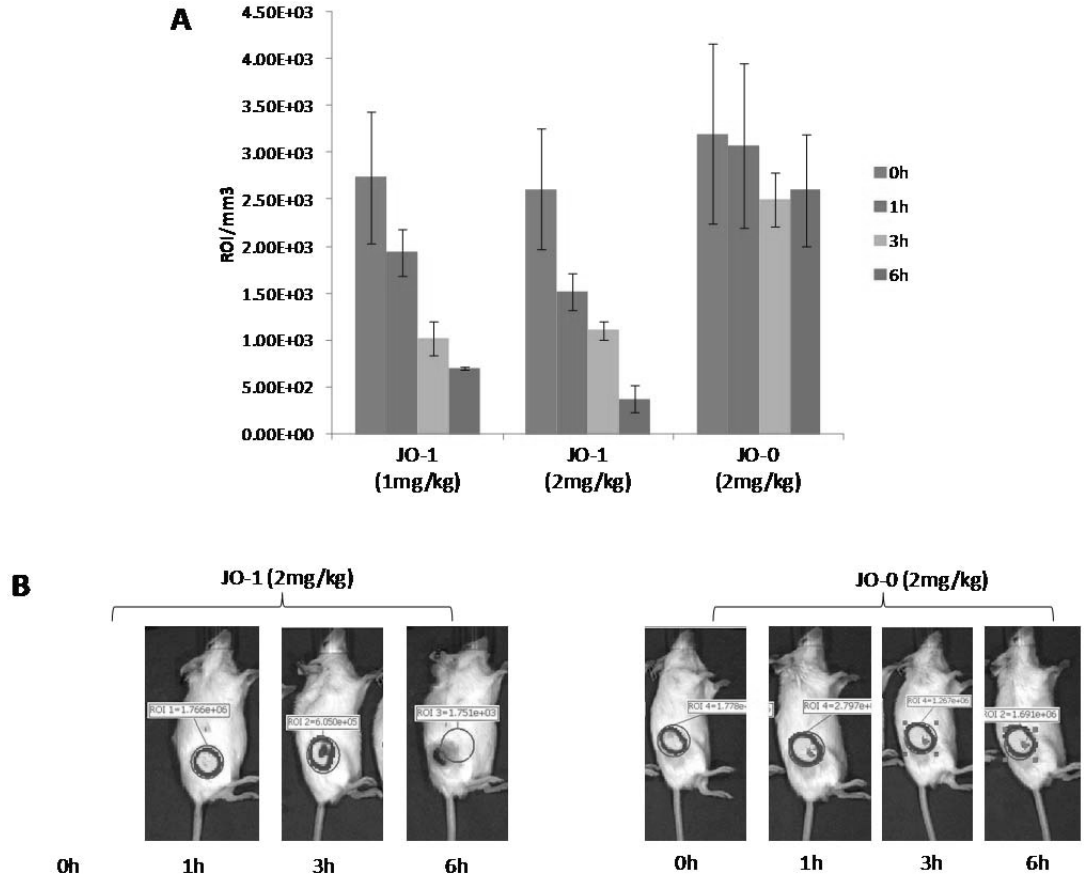


Figure 21. JO-1 decreases tumor hypoxia. Lung cancer A549 cells expressing luciferase under the control of a HIF-1 responsive promoter were subcutaneously injected into CB17-SCID/beige mice. When tumors reached a volume of 600mm^3, luciferase expression was analyzed by non-invasive imaging using an IVIS system. Images were taken 5 minutes before injection of JO-1 (1mg/kg or 2mg/kg) or JO-0 (2mg/kg) and 1, 3, and 6 hours after injection. **A)** Luciferase signals were normalized to the tumor volume (ROI/mm^3). N=5 **B)** Representative images.

4.4 Antibodies against JO-1 in human serum

As JO-1 is a viral protein, adaptive immune responses might develop in humans, particularly after repeated injection. Despite the fact that approximately one third of humans have neutralizing antibodies against Ad3 [1], our studies showed that these antibodies did not interact with JO-1 (Fig.22A). In a recent study, we did not find detectable antibodies against JO-1 in the serum of >60 ovarian cancer patients. Furthermore, polyclonal anti-JO-1 antibodies generated by vaccination of mice did not affect the enhancing effect of JO-1 transepithelial transport of PEG 4000 in polarized colon cancer cultures (Fig.22B). There are no detectable anti-JO-1 antibodies in serum of cancer patients. JO-1 remains active even in the presence of antibodies against JO-1.

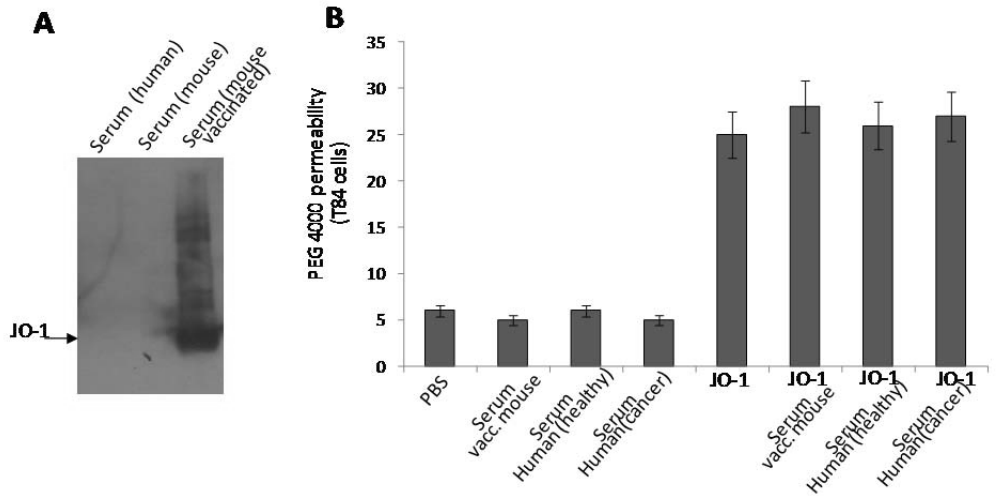


Figure 22. Sera from humans and hypervaccinated mice do not inhibit activity of JO-1. A) Analysis of serum for reactivity with JO-1 by Western blot. JO-1 was run on SDS-PAGE and filters were incubated with pooled human serum containing neutralizing antibodies against Ad3 virus (lane 1), serum from naïve mice (lane 2), or serum from JO-1 vaccinated mice (lane 3). **B)** JO-1 enhances transepithelial transport of PEG (4,000 Da) *in vitro* in the presence of anti-JO-1 antibodies. Pooled serum from 10 healthy donors ("healthy") and 10 breast patients ("cancer") or serum from a JO-1 vaccinated mouse ("vacc. mouse") was used in this study. Human colon epithelial T84 cells were incubated in transwell plates until transepithelial resistance was constant. JO-1 (5 μg/ml) or PBS mixed with heat-inactivated serum (at a final concentration of 20%) was added to the inner chamber, followed by ^{14}C-PEG-4,000 1 hour later. Radioactive counts were measured 1 hour later in the outer chamber. n=3

4.5 Studies in human DSG2 (hDSG2) transgenic mice

4.5.1 Human DSG2 functions in mouse tumor cells

JO-1 does not interact at a detectable level with mouse DSG2. This limits the value of safety studies in mice with human xenograft tumors. To test whether human DSG2 can interact with cell junction proteins and regulate epithelial junctions in mouse cells, we created a mouse mammary cancer (MMC) cell line [43], which ectopically expressed human DSG2 after lentivirus transduction. We called this cell line "MMC-hDSG2". Ectopic hDSG2 expression conferred infectability by Ad3-GFP (the virus from which JO-1 was derived from) (Fig.23A), whereby Ad3-GFP transduction could be blocked by recombinant soluble hDSG2 (Fig.23B). For further studies, MMC-hDSG2 cells were cultured under conditions that allowed for cell polarization. Studies on these cells were initiated once the transepithelial resistance was constant. Exposure of such MMC-hDSG2 cells to the hDSG2-ligand, JO-1, resulted in the partial removal of hDSG2 from epithelial junctions and in a partial dissolution of the junctions (Fig.23C). The finding that Ad3, PtDd, or JO-1 treatment of polarized MMC-hDSG2 cells cultured in transwell chambers increased the transepithelial transport of ^{14}C-PEG (MW = 4 kDa) (Fig.23D), supports the opening of junctions by JO-1. In control MMC cells (without hDSG2), Ad3 incubation did not increase the transepithelial transport compared to PBS treated cells (data not shown). The effect of JO-1 on MMC-hDSG2 cells is comparable to that seen on human cells [3,4,44]. MMC cells express rat *Neu* [43]. As seen in human breast cancer samples, rat *Neu* in MMC-hDSG2 cells appears to be trapped in the epithelial junctions (Fig.23E). JO-1 incubation of MMC-hDSG2 cells increases complement-dependent killing by a rat *Neu* specific monoclonal antibody (7.16.4-mAb) [45,46], most likely as a result of improved access to *Neu* (Fig.23F). These data suggest that human DSG2 in transgenic mice functions as a junction protein in a similar way as in human cells.

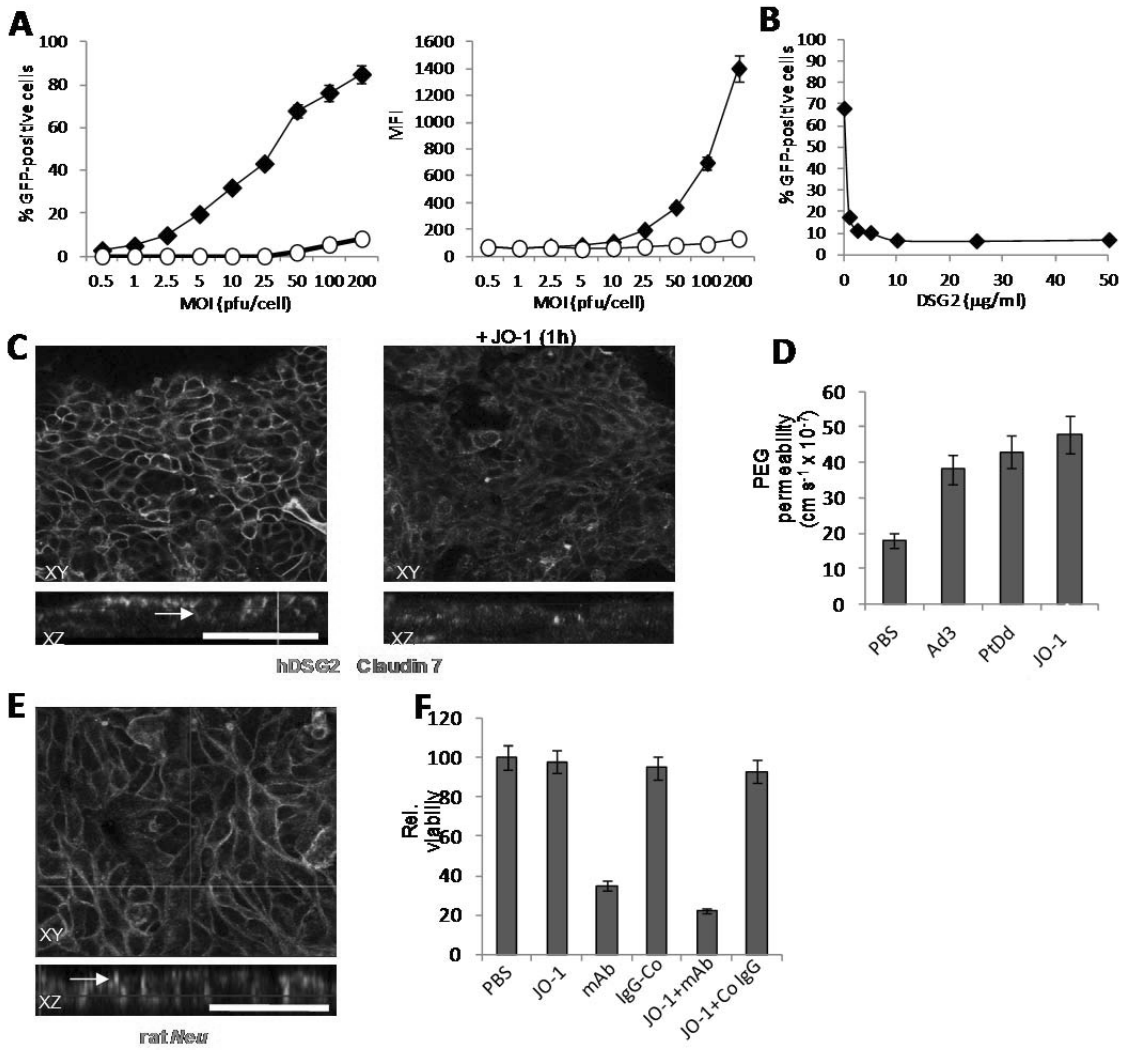


Figure 23. Function of human DSG2 in mouse mammary carcinoma cells expressing human DSG2 after lentivirus gene transfer (MMC-hDSG2 cells). A) Transduction of MMC cells (empty circles) and MMC-hDSG2 cells (black diamonds) by Ad3-GFP at increasing MOIs. The percentage of GFP-expressing cells and mean fluorescence intensity (MFI) was analyzed 18 hours after infection. N = 3. **B)** Competition of Ad3-GFP infection by recombinant, soluble hDSG2. MMC-hDSG2 cells were incubated with increasing concentrations of hDSG2 for 60 min before being infected with Ad3-GFP vectors at an MOI of 100 pfu/cell for 60 min, after which the viruses were removed and new medium was added. GFP fluorescence was measured 18 hours later. N = 3. Shown are average values. The standard deviation was less than 10% for all samples. **C)** Confocal immunofluorescence microscopy of MMC-hDSG2 cells before and 12 hours after exposure to 0.5 µg/ml of JO-1. The scale bar is 40 µm. The white arrow in the XZ panel indicates an area with junction-localized DSG2. **D)** ^{14}C-PEG-4,000 diffusion through monolayers of MMC-hDSG2 cells at 30 min after adding inactivated Ad3 virions (Ad3) ($2x10^8$ particles/ml), Ad3 penton-dodecahedra (PtDd) (0.5µg/ml), or JO-1 (0.5µg/ml). N = 5. **E)** Confocal immunofluorescence microscopy of MMC-hDSG2 cells. Rat Neu (green) appears to be trapped in junctions (see white arrow). **F)** JO-1 enhances killing of MMC-hDSG2 cells by the Neu-specific monoclonal antibody 7.16.4-mAb. MMC-hDSG2 cells were incubated with 0.5 µg/ml JO-1 or PBS followed by 7.16.4-mAb ("mAb") or control antibody ("IgG-Co") 12 hours later. Viability was measured 4 days later. Viability of PBS treated cells was taken as 100%, N = 5. $p < 0.05$ for mAb vs. JO-1+mAb.

4.5.2 **Generation of human DSG2 transgenic mice**

To confer tissue-specific human DSG2 expression in transgenic mice, we used the complete (~90 kb) human DSG2 locus, including its endogenous regulatory elements (Fig.24A). Expression of human DSG2 in skin epidermis of transgenic mice was confirmed by immunohistochemistry on tail and ear sections. Tail skin fibroblasts from both animals were cultured and metaphase chromosomes were subjected to DNA fluorescence in situ hybridization using the human DSG2 locus as a probe (Fig.24B.) For founder mouse #187, we found the hDSG2 signal associated with one of the 40 total chromosomes, suggesting one integration site. We employed an inverse PCR approach on genomic DNA isolated from fibroblasts to map the transgene/mouse genome junctions in founder #187 (which we called hDSG2-transgenic mice). The transgene was inserted into position 81,491,601 of mouse chromosome 16 (Fig.24C). The insertion site is located within the second intron of the neural cell adhesion molecule 2 (NCAM2) gene. Previous studies have shown that knockout of this gene has no critical side effects [47]. These studies also detected the presence of a head-to-tail concatemer. Consistent with this, we measured two transgene copies per genome by hDSG2 qPCR on genomic DNA using a standard curve based on the human DSG2 BAC.

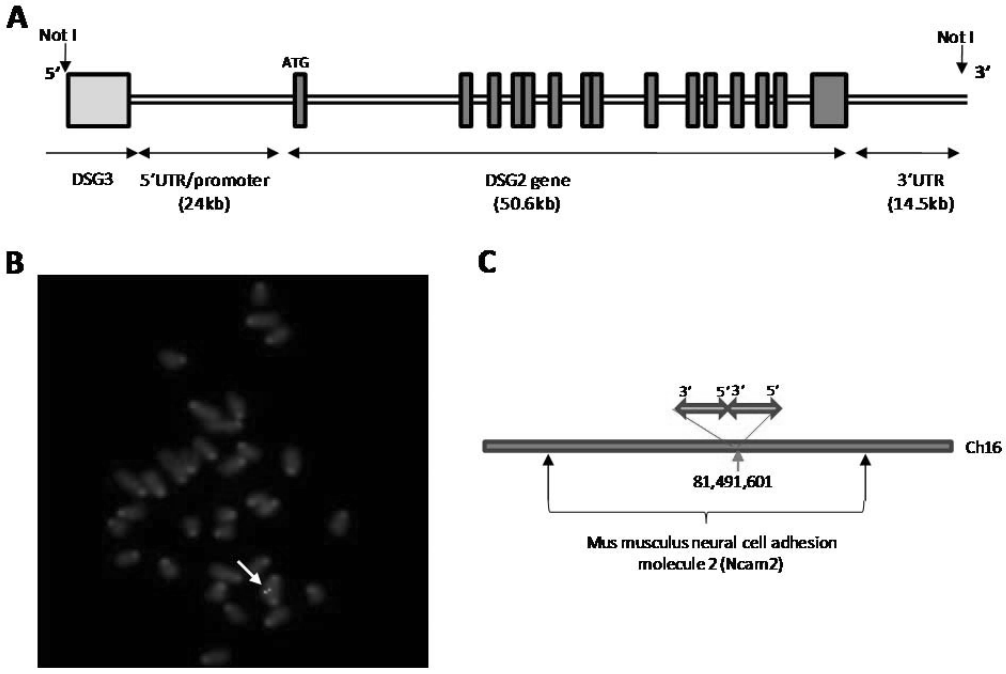


Figure 24. Characterization of hDSG2 transgenic mice. A) The Bacterial Artificial Chromosome **(**BAC) (Invitrogen CTD-223319) used for generation of transgenic mice contains a 89.17 kb fragment of human chromosome 18 from position 29,054,153 to 29,143,320. The fragment contains the 24 kb DSG2 promoter and 5'untranslated regions (UTR), the 50.6 kb DSG2 gene, and 14.5 kb of the 3'UTR. **B)** Analysis of mouse strain 187. Fluorescence *in situ* hybridization for hDSG2 on fibroblasts from hDSG2 transgenic mice. Cultures of tail fibroblasts were treated with colcemide and metaphase chromosomes were hybridized with the hDSG2 BAC labeled with Cy3. Positive signals appear as green "snake eyes" (see arrow). **C)** Integration site analysis of mouse strain 187. Genomic DNA from tail fibroblasts was subjected to inverse PCR analysis and transgene/chromosome junctions were sequenced. The human DSG2 locus is inserted as a head-to-tail tandem into mouse chromosome 16 position 81,491,601 without chromosomal rearrangements. The insertion site is within the second intron of the mouse Ncam2 (neural cell adhesion molecule 2) gene. The presence of two transgene copies per diploid genome was confirmed by qPCR for the human DSG2 gene.

4.5.3 **Biodistribution of hDSG2 expression in transgenic mice is similar to that in humans**

Human DSG2 mRNA in tissues of hDSG2-transgenic mice was measured by qRT-PCR and mRNA levels were compared to hDSG2 mRNA levels in skeletal muscle, the tissue with the lowest hDSG2 expression. Both the distribution and level of hDSG2 mRNA expression in transgenic mice were similar to those in humans (Fig.25A). In immunohistochemistry analysis, marked hDSG2 signal expression was seen in the GI-, respiratory, and urinary tract epithelium. hDSG2 was found in the intercalated discs of the myocardium, in pancreatic ducts, and in the choroid plexus of the brain (Fig.25B). Flow cytometric studies on blood cells from hDSG2-transgenic mice revealed that 11.6+/-1.8% platelets were hDSG2 positive. 46+/-12% of leukocytes stained positive for hDSG2. The concentration of hDSG2 in serum of hDSG2- transgenic mice was 1230+/-190 ng/ml. hDSG2 was not detectable in serum and on blood cells of non-transgenic littermates.

<u>In summary, these data demonstrate that hDSG2-transgenic mice have a similar distribution of hDSG2 to humans. Futhermore, JO-1 binding to human DSG2 expressed in mouse cells triggers junction opening. Therefore, hDSG2-transgenic mice are an adequate model to study JO-1 biodistibution and safety.</u>

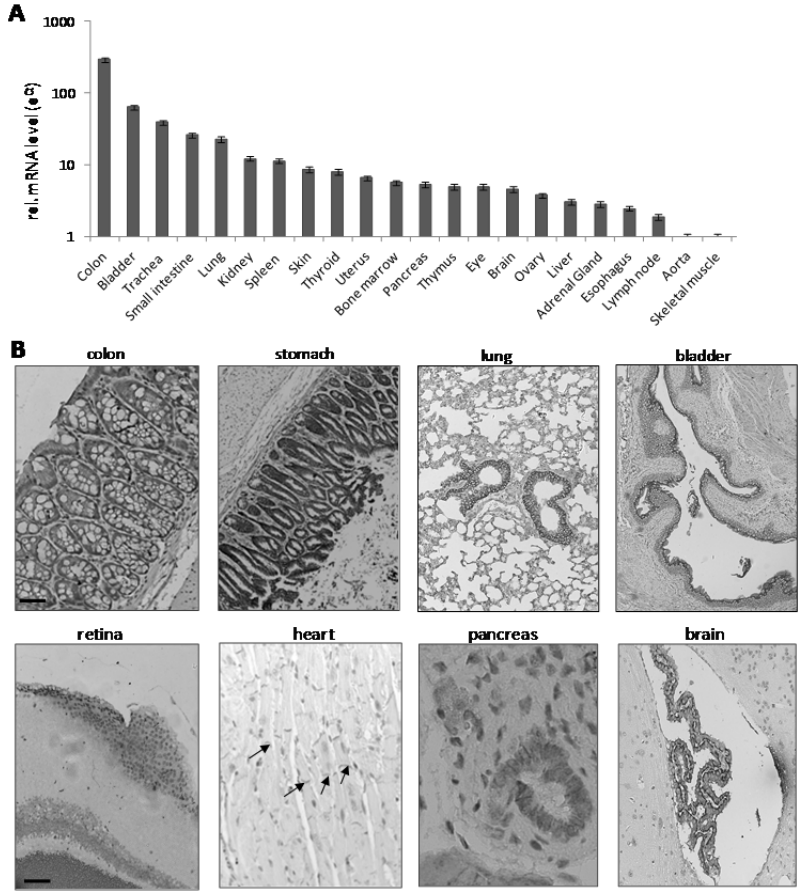


Figure 25. hDSG2 expression in tissues of hDSG2 transgenic mice. A) hDSG2 mRNA expression. Total RNA was isolated, reverse transcribed, and qRT-PCR was performed. Shown are relative hDSG2 mRNA levels. The $e^{\Delta CT}$ value of skeletal muscle was taken as "1". N = 3 (different animals). Notably, hDSG2 mRNA background signals in non-transgenic littermates were below the levels in skeletal muscle of hDSG2 transgenic mice. **B)** hDSG2 immunohistochemistry analysis of paraffin sections. hDSG2 appears in brown. Sections are counterstained with hematoxylin. Representative sections are shown. The scale bars are 20 μm. Examples of hDSG2 staining in intercalated discs in the myocardium are labeled by arrows.

4.5.4 **Biodistribution and serum half-life of JO-1 in hDSG2 transgenic mice**

These studies were performed in hDSG2 transgenic mice with MMC-hDSG2 tumors. JO-1 binds to MMC-hDSG2 cells and triggers reorganization of junctions in a similar way as seen in human epithelial cells, indicating that JO-1 signaling through hDSG2 can override junction regulation in mouse cells [48]. Transplantation of MMC-hDSG2 cells into the mammary fat pad of hDSG2 transgenic mice resulted in tumors that resembled key features of breast cancer in humans, e.g. nests of hDSG2^{bright} epithelial cells that were surrounded by tumor stroma (Fig.26A). Intravenous injection of JO-1 resulted in efficient accumulation of JO-1 in MMC-hDSG2 tumor nests as seen on tumor sections analyzed 6 hours after injection (Fig.26A). Immunoreactive JO-1 was also found in epithelial cells of the intestine in hDSG2 transgenic mice (Fig.26B), which is consistent with our recent biodistribution study with Ad3-GFP virus [48]. We measured JO-1 concentrations in tissue and tumor lysates by ELISA at 6 hours after injection in the following 4 groups of animals: *i)* hDSG2-transgenic mice with MMC-hDSG2 tumors (Fig.26C "hDSG2-pos"), *ii)* hDSG2-transgenic mice without tumors (Fig.26D "hDSG2-pos"), *iii)* non-transgenic littermates with MMC tumors (Fig.26C "hDSG2-neg"), and *iv)* non-transgenic littermates without tumors (Fig.26D "hDSG2-neg"). We found highly selective, hDSG2-dependent accumulation of JO-1 in MMC-hDSG2 tumors established in hDSG2 transgenic mice. About 10-fold less JO-1 was found in the intestine and colon of hDSG2 transgenic mice. JO-1 was also detected in the liver and spleen of both hDSG2-positive and –negative mice, suggesting an hDSG2 independent uptake. The amount of JO-1 in the intestine and colon was more than 2-fold higher in hDSG2 transgenic mice without MMC-hDSG2 tumors, supporting our hypothesis that the tumor acts as a "sink" for JO-1. From the JO-1 biodistribution studies in MMC-hDSG2 tumor-bearing hDSG2 mice, the question arose why JO-1 predominantly accumulates in the tumor. Immunofluorescence studies on tumor and intestine sections of hDSG2 transgenic mice, showed that epithelial tumor cells lack strict polarization, in contrast to normal epithelial cells (Figs. 26E and F). As a consequence, in tumors, hDSG2 appears to be readily accessible, while in intestinal epithelial cells most of the hDSG2 is trapped in junctions, reflected by overlapping signals for hDSG2 and the junction marker claudin 7 (Fig.26F).

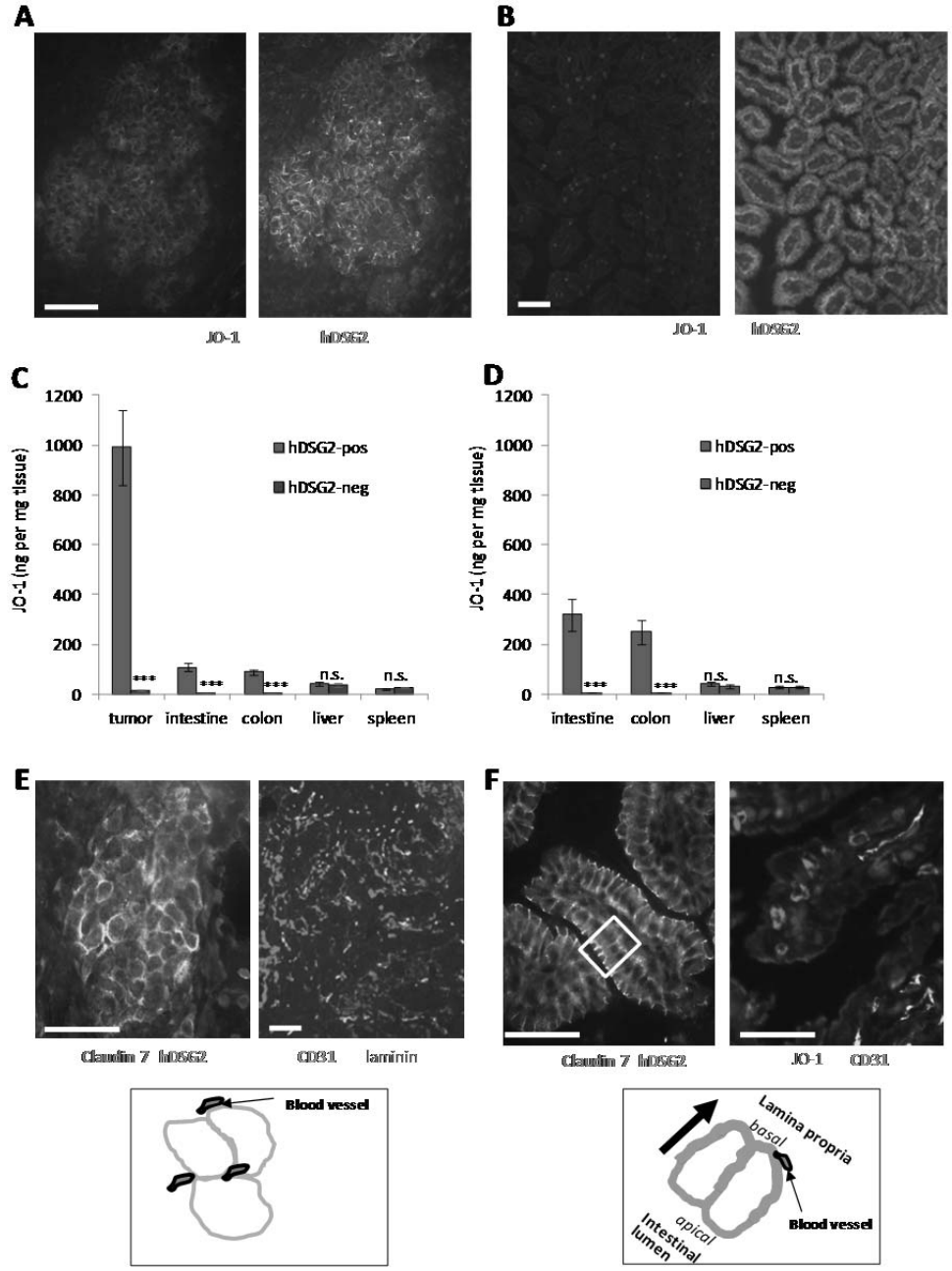


Figure 26. JO-1 biodistribution studies in hDSG2 transgenic mice. A-C) A total of $4x10^6$ MMC-hDSG2 cells were injected into the mammary fat pad of hDSG2 transgenic mice. When tumors reached a volume of 100 mm^3, JO-1 (2 mg/kg) was injected intravenously. Mice were sacrificed 6 hours later, blood was removed from the circulation, and organs were harvested. **A and B)** Sections of MMC-hDSG2 tumors and intestine stained for JO-1 (red) and hDSG2 (green). **C and D).** JO-1 biodistribution: JO-1 (2 mg/kg) was injected intravenously. Six hours later, blood was flushed from the circulation and tissue lysates were analyzed for JO-1 by ELISA. n=3. ***P<0.001, n.s. non-significant. **E and F)** Tumor (E) and intestine (F) sections from MMC-hDSG2 tumor bearing hDSG2 transgenic mice were stained for the adherens junction marker claudin 7 (red) and hDSG2 (green). Representative sections are shown. The scale bars are 20 μm.

Immunohistochemistry analyses, showed that JO-1 in the liver is mostly present in sinusoidal spaces and F4/80-positive Kupffer cells (Fig. 27) and not taken up by parenchymal liver cells. Similarly, in the spleen, JO-1 signals appear in the peripheral zone of germinal centers (Fig.27) and are mostly likely the result of uptake by splenic macrophages.

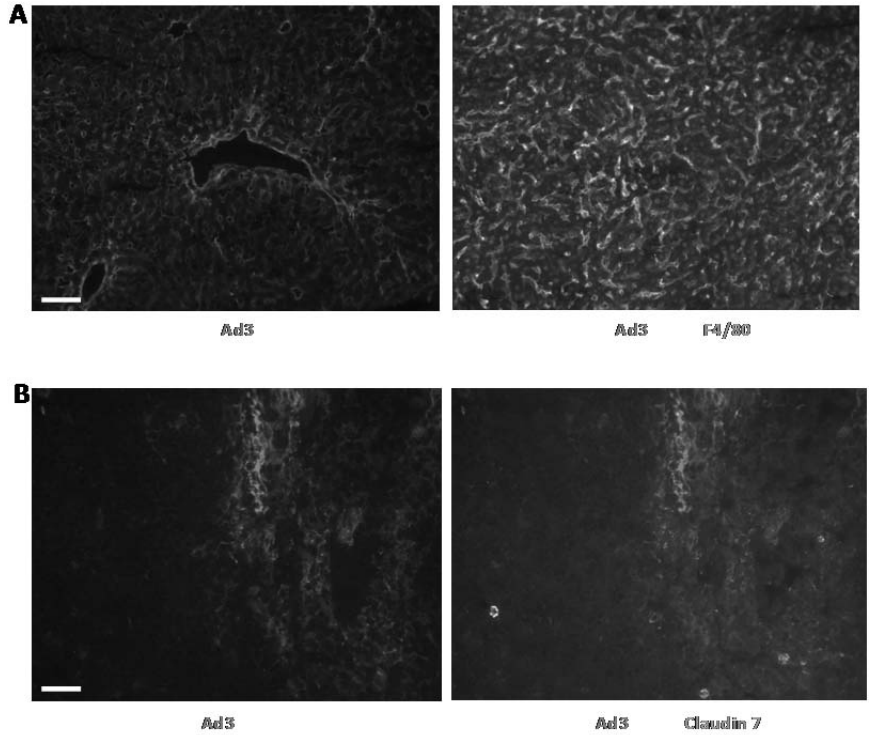


Figure 27. JO-1 in liver and spleen. hDSG2 transgenic mice were injected with JO-1 (2mg/kg). Organs were harvested 6 hours later. A) Staining of liver sections for Ad3 particles (red) and the Kupffer cell marker F4/80 (green). B) Staining of spleen sections for Ad3 particles (red) and the adherens junction marker claudin 7 (green). Representative sections are shown. The scale bars are 20μm.

We also measured clearance of JO-1 from blood after intravenous injection (Fig.28). These studies show that the half-life of JO-1 in hDSG2 transgenic mice is approximately 6 hours. JO-1 clearance from blood is slower in hDSG2 transgenic mice than in non-transgenic littermates, which could be due to the fact that hDSG2 is expressed on platelets and subfractions of leukocytes [48].

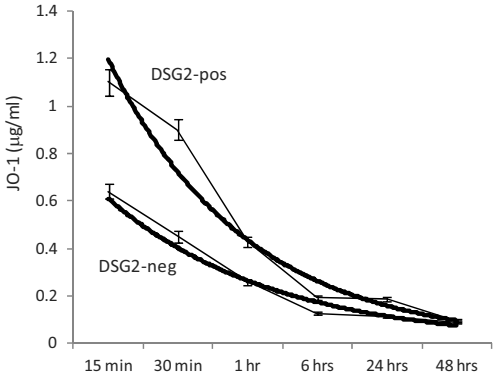


Figure 28. Serum clearance of JO-1. hDSG2 transgenic mice (hDSG2-pos.) or nontransgenic littermates (hDSG2-neg.) were intravenously injected with JO-1 (2mg/kg) and serum samples were analyzed for JO-1 by ELISA. N=3.

In summary, studies in hDSG2 transgenic mice show that JO-1 has a half-life in blood of ~6hours. After intravenous injection JO-1 is preferentially taken up by DSG2-positive tumors. A number of factors could account for this, including *i)* overexpression of DSG2 on tumor cells, *ii)* better accessibility of DSG2 on tumor cells because of a lack of strict cell polarization, and *iii)* a high degree of vascularization and vascular permeabilty in tumors. Small amounts of JO-1 were taken up by intestinal epithelial cells.

4.5.5 **Therapy studies of a combination of JO-1 and PLD in hDSG2 transgenic mice**

To test the potential for anti-JO-1 antibody responses to adversely affect the therapeutic effects of JO-1, we performed repeated injections of JO-1 in an immunocompetent hDSG2 mouse tumor model (Fig.29). After two treatment cycles of JO-1 and PLD, treatment was stopped and tumors were allowed to re-grow. The 3rd and 4th treatment cycles were started on days 28 and day 35, respectively. At the time of the 3rd cycle, serum anti-JO-1 antibodies were detectably by ELISA in the JO-1/PLD treated group of animals. However, the antibody levels were about ~10-fold lower than in the group, which received JO-1 without PLD. Importantly, in both, the 3rd and 4th treatment cycles JO-1 had an enhancing effect on PLD therapy, demonstrating that JO-1 continues to be effective after multiple treatment cycles, even in the presence of detectable antibodies. This may be due to the fact that JO-1 binds to DSG2 with a very high avidity thus disrupting potential complexes between JO-1 and anti-JO-1 antibodies. (Notably JO-1 is a dimer of a trimeric fiber knob, which contributes to the picomolar avidity to DSG2 [3].)

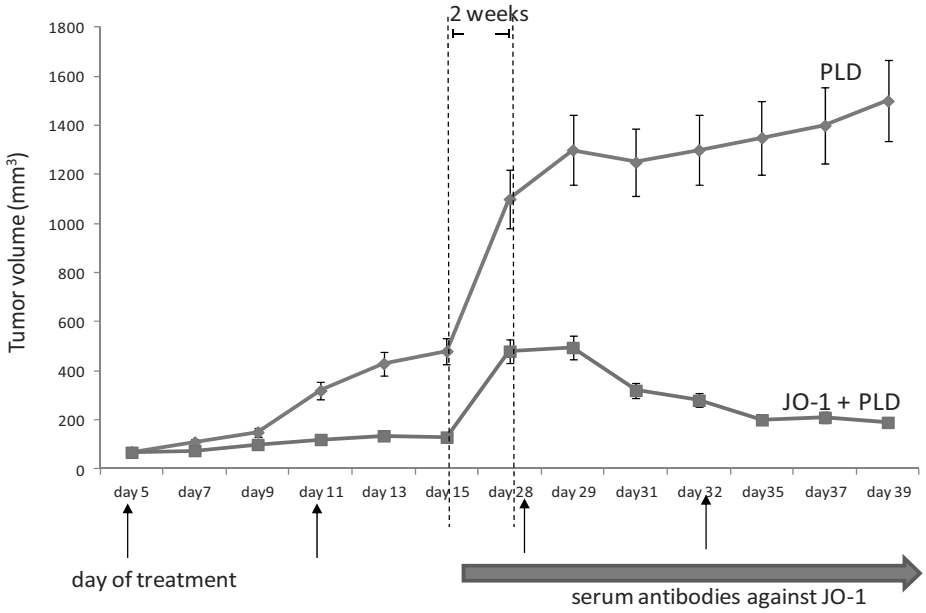


Figure 29. JO-1 plus PLD/Doxil therapy in immuno-competent hDSG2 transgenic mice. A total of 4x10[6] TC1-hDSG2 cells were injected into the mammary fat pad of hDSG2 transgenic mice. When tumors reached a volume of ~80mm[3], JO-1 (2mg/kg) or PBS was injected intravenously followed one hour later by PLD/Doxil (i.v. 1.5mg/kg). Treatment was repeated as indicated by arrows. Tumors were then allowed to re-grow for about two weeks. From day 15 on serum anti-JO-1 antibodies were detectable. Two more treatment cycles were performed at day 28 and day 35. N=5. JO-1 continues to be effective after multiple treatment cycles, even in the presence of detectable antibodies.

4.5.6 **Safety studies in hDSG2 transgenic mice**

Except for a mild, transient diarrhea, intravenous injection of JO-1 (at a dose of 2mg/kg) into hDSG2 transgenic mice had no other observed adverse effects, and there were no abnormalities found in laboratory parameters, including hematologic and serum chemistry parameters as well as histopathological studies of tissues. We speculate that this is due to the fact that hDSG2 in tissues other than the tumor and a subset of epithelial cells in the intestine/colon is not accessible to intravenously injected JO-1. Fig.30 shows that there were no significant changes in blood parameters at 24 hours (Fig.30A and B) and 3 days (not shown) after injection of JO-1 at a dose of 2mg/kg, i.e. a dose that conferred an enhancing effect on Doxil therapy. After injection of JO-1 at a dose of 10mg/kg, blood analysis detected leucopenia, decrease in serum glucose levels, and increase in serum GPT levels. Liver damage is also reflected by the presence of steatosis in the liver (Fig.30C). Notably, the potential danger of provoking a serious immune response as a consequence of the administration of multiple doses of JO-1 to patients has to be taken into consideration.

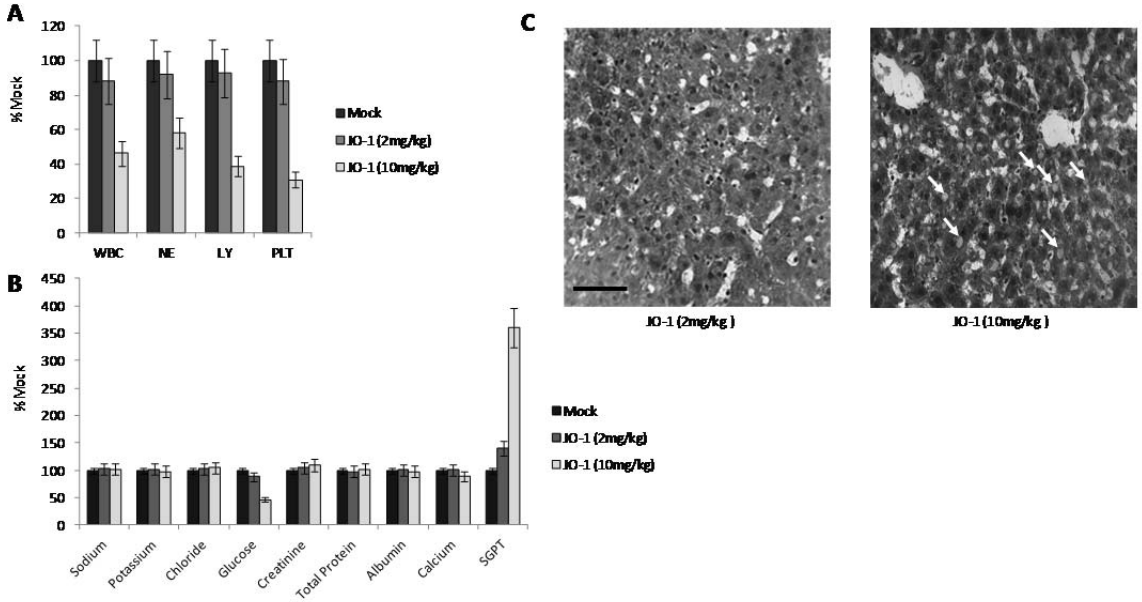


Figure 30. JO-1 safety studies in hDSG2 transgenic mice. hDSG2 transgenic mice were intravenously injected with JO-1 at 2mg/kg and 10mg/kg. Twenty four hours later, mice were sacrificed. **A)** Analysis of White blood cells (WBC), neutrophils (NE), lymphocytes (LY) and platelets (PLT). N=3. **B)** Analysis of blood chemistry. SGPT=ALT. n=3. **C)** H&E staining of liver sections. Fat droplets are indicated by arrows. The scale bar is 20μm.

Pathological mechanism that lead to mild diarrhea. Intravenous injection of JO-1 caused a mild diarrhea in mice within hours after intravenous infusion. Animals were back to normal by 24 hours after JO-1 injection. The mechanism behind this immediate/acute diarrhea included the following subsequent steps [49]: JO-1 is taken up by rare intestinal epithelial cells, which triggers opening of epithelial junctions and increase in intestinal permeability. Influx of intestinal bacteria or bacterial antigens will elicit TLR6-mediated activation of expression of pro-inflammatory cytokines and chemokines which, in turn, will triggers intestinal injury. This is supported by our studies Ad3 the virus JO-1 is derived from. At two hours after Ad3-GFP injection, levels of key pro-inflammatory cyto/chemokines IL-1α, IL-1β, IL-2, IL-27, MIG/CXCL9, MIP-1a/CCL3, and TNFα were significantly ($p < 0.05$) higher in intestine lysates of hDSG2-transgenic mice when compared to hDSG2-negative mice (Fig.31 and B). Levels of the anti-inflammatory cytokine IL-10 were lower in hDSG2-transgenic mice. Of particular interest is the elevation of IL-1□,

TNFα, and MIP1α expression, which is usually activated through Toll-like receptors (TLRs), specifically TLR2 and TLR6. TLR2 and 6 are in turn activated in epithelial or dendritic cells/macrophages upon recognition of bacterial or mycoplasma lipoproteins [50]. In this context, we found stronger TLR6 signals in intestinal epithelial cells of Ad3-GFP injected hDSG2-transgenic mice than in control mice (Ad3-GFP injected hDSG2-negative mice or mock-injected hDSG2 transgenics) (Fig.31B). These findings support a potential etiological link between JO-1 injection and diarrhea.

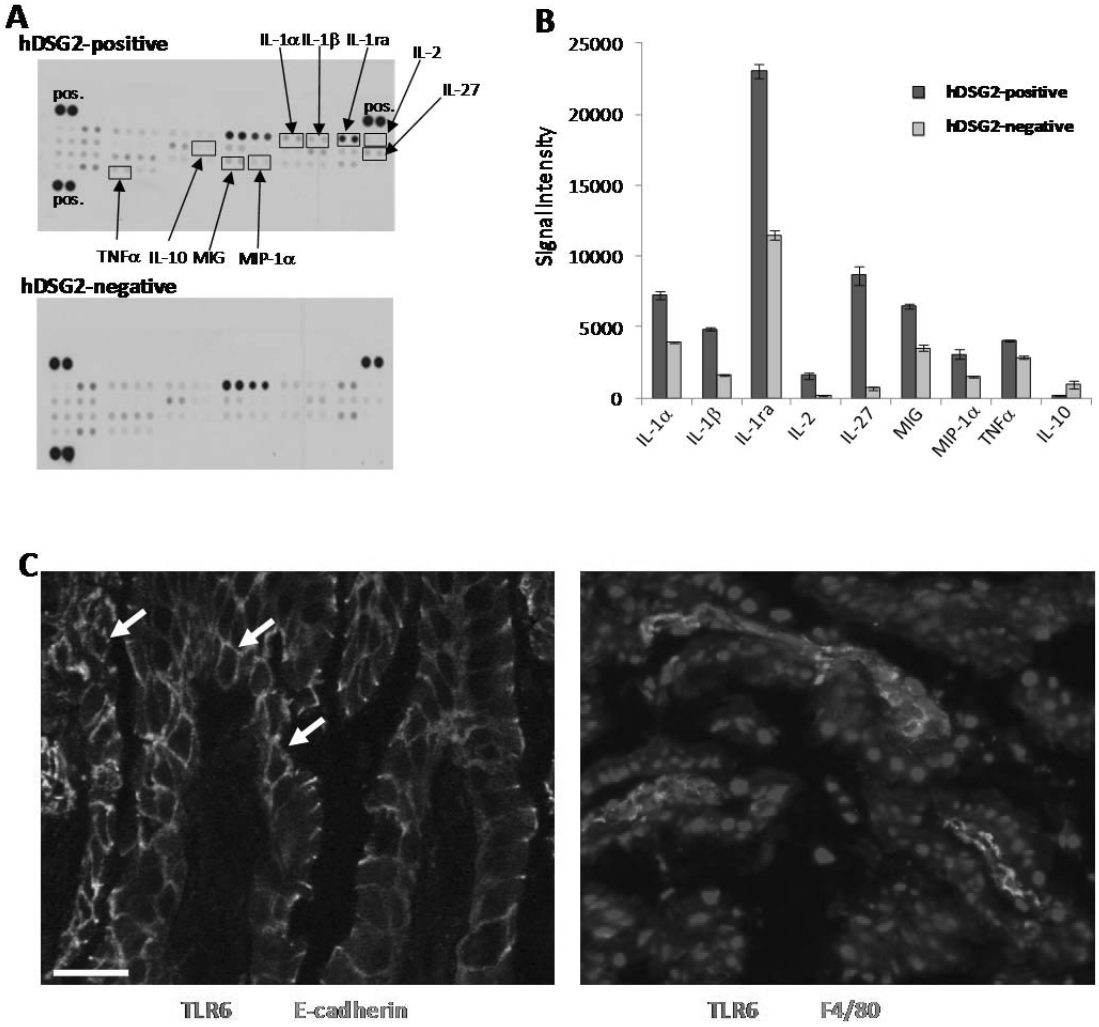


Figure 31. Activation of cytokines and TLR following intravenous Ad3-GFP. A and B) Cytokine array. Small intestine and colon samples were combined and lysed. Lysates were analyzed for a set of 40 mouse cytokines and chemokines using the Proteome Profiler Array kit from R&D Systems. N = 3. **A)** Representative image of dot blot from tissue of one mouse per group. **B)** Quantification of signals from all animals (N = 3). The blots were scanned and signal intensity was measured. The array includes positive controls ("pos.") provided by the manufacturer. Cyto-/chemokines that significantly differ in their concentrations ($p < 0.05$) in both groups are highlighted in the upper dot blot. MIG = CXCL9, MIP-1 = CCL3. (Notably, as antibodies specific for each individual cytokine are used in this cytokine array, absolute levels for different cytokines cannot be compared. This implies that the levels of IL-1ra, a protein that inhibits IL-1α and –β activity, might be lower than those of IL-1α and IL-1β.) **C)** Immunofluorescence analysis on intestine sections of hDSG2 positive mice for TLR6 and E-cadherin (left panel) and TLR6 and F4/80 (right panel). The scale bar is 20μm. Representative sections are shown.

Overall, our studies in hDSG2 transgenic mice show that JO-1 (2mg/kg) co-therapy is safe and effective in the presence of anti-JO-1 antibodies.

4.5.7 **Effect of JO-1 on anti-tumor T-cell responses**

In this study, we used hDSG2 transgenic mice and syngeneic TC1 cells that expressed human DSG2 (Fig.32). These mice are immunocompetent, in contrast to the immunodeficient mice used in the human xenograft models described above. TC1 cells also express HPV E6 and E7 and trigger E7-specific T-cells. However, these tumor-specific T-cells are unable to control tumor growth and 100% of tumor-bearing animals reach the study endpoint by day 20. Two injections of JO-1 into tumor-bearing mice at days 7 and 14 resulted in tumor regression in 60% of mice (Figs.32A and B). This effect was due to an increased number of E7-specific T-cells inside the tumor (Figs.32C and D), which could be blocked by systemic depletion of CD8 cells (Fig.33A). JO-1 had no effect on tumor growth in immunodeficient mice (Fig.33B). Potential explanations for the observed effect are: i) increase of intratumoral penetration of anti-tumor T-cells, ii) relocalization and better accessibility of target antigens on tumor cells, iii) JO-1-mediated decrease of hypoxia and subsequent chemotaxis and activation of Tregs. We are currently performing studies to evaluate these hypotheses. It is relevant for the clinical trial to note that JO-1 not only increased the frequency of tumor-specific T-cells in the tumor, but also in the periphery, e.g. the spleen. This observation warrants the analysis of anti-tumor T-cells in the peripheral blood of patients.

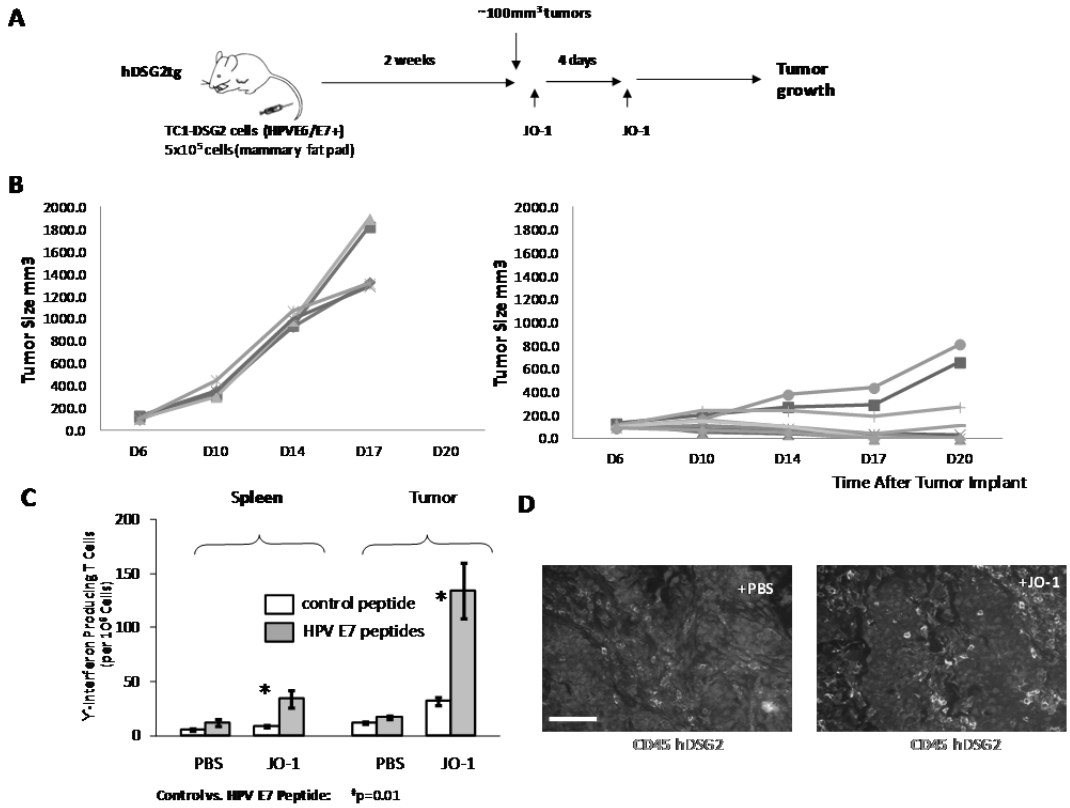


Figure 32. JO-1 facilitates anti-tumor T-cell responses. A and B) hDSG2 transgenic mice with established subcutaneous TC1-hDSG2 tumors were intravenously injected with PBS or JO-1 (2mg/kg) at day 6 and 10 after tumor cell transplantation and tumor volumes were measured. Each line represents an individual animal. **C)** Analysis of frequencies of IFNγ-producing E7 specific T-cells in the spleen and tumor 2 days after PBS or JO-1 injection (at day 14 p.i.). Shown are the frequencies of IFNγ producing T-cells specific to the HPV16 E749-57

carrying the H-2Db restricted peptide (RAHYNIVTF) or an unrelated control peptide ("Control). N=3 animals per group. The differences in the JO-1 group are significant ($p<0.05$). D**)** TC1-hDSG2 tumor sections 2 days after PBS or JO-1 injection stained for hDSG2 (red) and the pan-leukocyte marker CD45 (green). The scale bar is 20μm. The number of CD45-positive cells in tumor nests was 7.8(+/-3.4) cells per mm^2 in PBS injected mice and 35+/-12 cells per mm^2 in JO-1 injected mice (n=3).

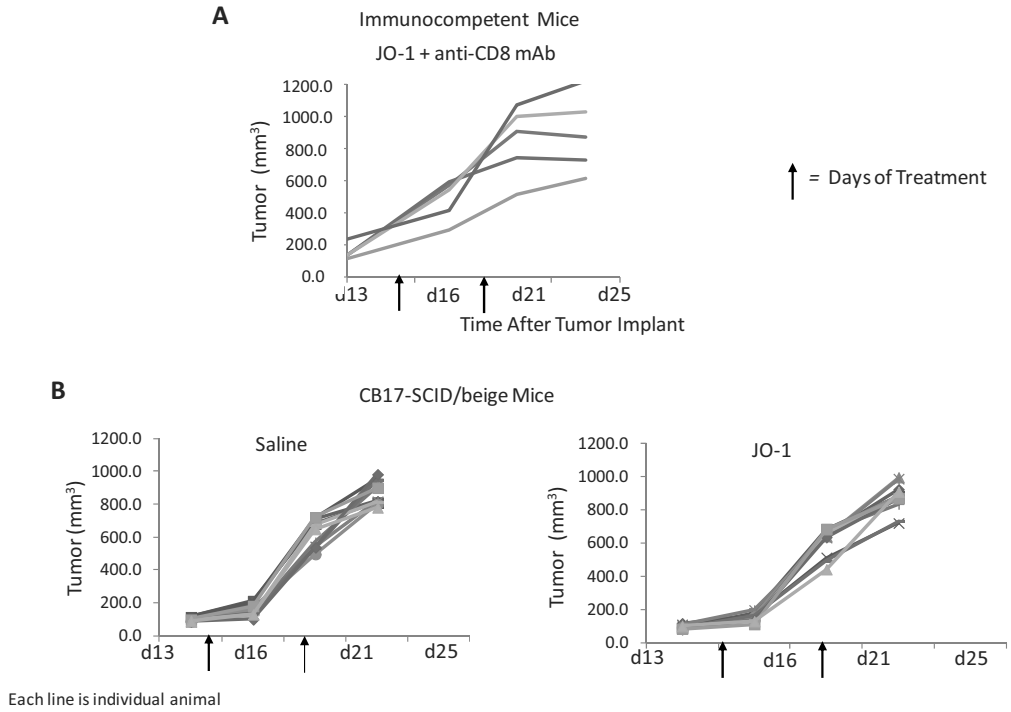


Figure 33. JO-1 effect is T-cell mediated. Studies were performed as described in Fig.32. **A)** $CD8^+$ T cells were depleted using i.p. injection of 200 μg rat anti-mouse CD8 IgG (169.4; ATTC). **B)** TC1-hDSG2 tumors were implanted into immunodeficient CB17-SCID/beige mice.

4.6 Safety studies with JO-1 in non-human primates

4.6.1 **Macaca fascicularis as an adequate model for JO-1 toxicology studies**

The hDSG2 transgenic mouse model allowed us to test many parameters of JO-1 therapy and safety in a large number of animals. However, it is unclear whether the hDSG2-mouse system completely models a homologous system with human DSG2 in human cells. A better model is non-human primates. DSG2 biodistribution in the tested NHP species, ***Macaca fascicularis***, is nearly identical to that in humans, and JO-1 binds to monkey DSG2 [48].

Unlike rodent DSG2, we demonstrated that Ad3 utilized monkey DSG2 for infection (Figs. 34A and B). African Green Monkey (Vero) cells can be efficiently transduced by Ad3-GFP and this transduction is mediated by monkey DSG2. Monkey DSG2-specific siRNA-mediated knockdown of monkey DSG2 expression decreases the transduction efficiency of Ad3-GFP, but not that of the CD46-interacting Ad5/35-GFP or CAR-interacting Ad5-GFP vector (Fig.34A). The hDSG2 ligand JO-1 specifically and efficiently blocked Ad3-GFP infection of Vero cells (Fig.34B) and increased transepithelial permeability in polarized Vero cell monolayers (data not shown).

qRT-PCR for monkey DSG2 mRNA was performed on total RNA isolated from *Macaca fascicularis* tissues. Both frozen and paraffin sections were analyzed for DSG2 immunoreactivity. Overall, the pattern of

mRNA expression was similar to that seen in humans (Fig.34C). The use of frozen tissues sections enabled us to perform co-staining of monkey DSG2 and the epithelial junction protein E-cadherin (Fig.34D). Selected images show the localization of DSG2 in epithelial junctions of the GI and respiratory tracts, as well as in intrahepatic bile ducts and prostatic acini. In addition, we demonstrated the presence of DSG2 in lung and cornea epithelium (Fig.35A) and in salivary glands, pancreatic ducts and dermal sweat glands (Fig.35B). DSG2 was also detected in intercalated discs of myocardiac tissue (Fig.35C). In the brain, DSG2 was observed in the choroid plexus (Fig.35D) but not in endothelial junctions forming the blood brain barrier (Fig.35D, right panel). In agreement with human studies we found DSG2 expression on ~80% of macaque bone marrow cells and on 27.7+/-6% of platelets. The fraction of DSG2+ leukocytes (CD45+) was 25.9+/-8% and therefore higher than in humans (data not shown).

Overall, our analyses of monkey tissue suggest that non-human primates express DSG2 in a pattern similar to humans. Therefore NHP are an adequate model for safety studies with JO-1.

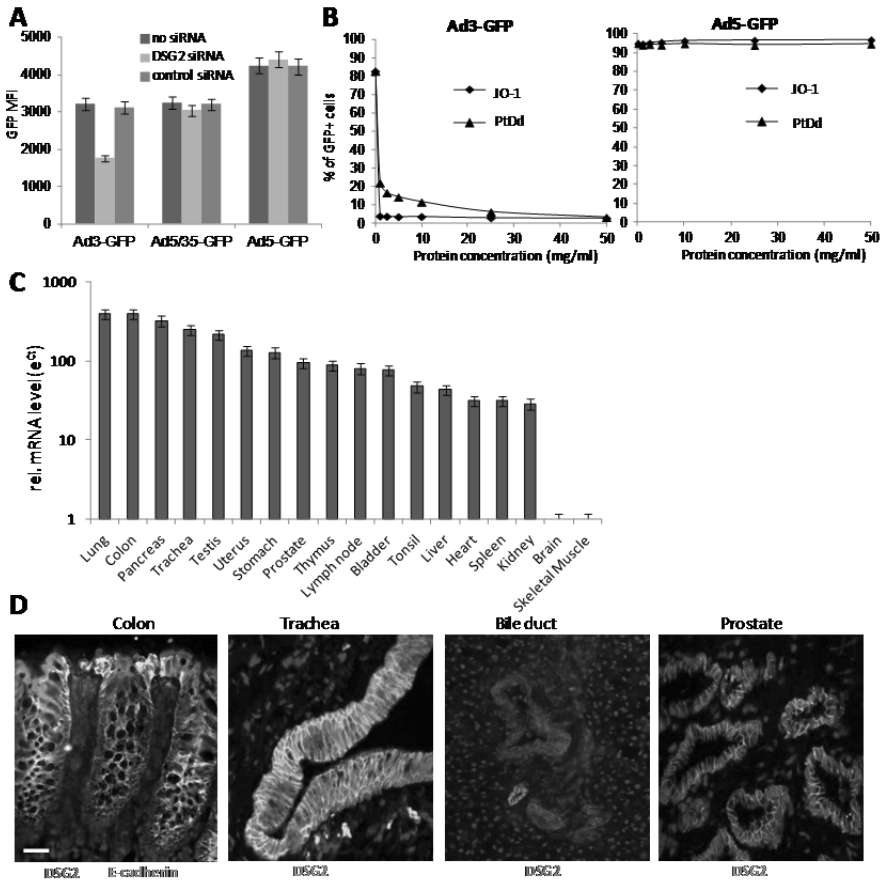


Figure 34. Ad3 transduction of monkey cells and DSG2 expression in tissues of *Macaca fascicularis*. A and B) Transduction studies in Green Monkey Vero cells. **A)** siRNA knockdown of monkey DSG2 expression. Vero cells were transfected with monkey DSG2-siRNA or control siRNA and, 48 hours later, infected with Ad3-GFP, Ad5/35-GFP, or Ad5-GFP vectors. GFP expression was analyzed 18 hours after infection. **B)** Vero cells were incubated at increasing concentrations of the DSG2 ligands JO-1 or Ad3 penton-dodecahedra (PtDd) for one hour and then infected with 100 pfu/cell of Ad3-GFP or Ad5-GFP virus. Shown is the percentage of GFP expressing cells measured 18 hours after infection. **C)** Monkey DSG2 mRNA expression. Total RNA was isolated, reverse transcribed, and qRT-PCR was performed. Shown are relative DSG2 mRNA levels. ΔCT values were normalized for GAPDH expression and the log $e^{\Delta CT}$ was used to compare DSG2 mRNA levels between tissues, whereby the value for skeletal muscle was

taken as 1. N = 3 (technical replicates). **D)** Monkey DSG2 immunofluorescence analysis of macaque tissue. The colon section is also co-stained for E-cadherin. Representative sections are shown. The scale bar is 20 μm.

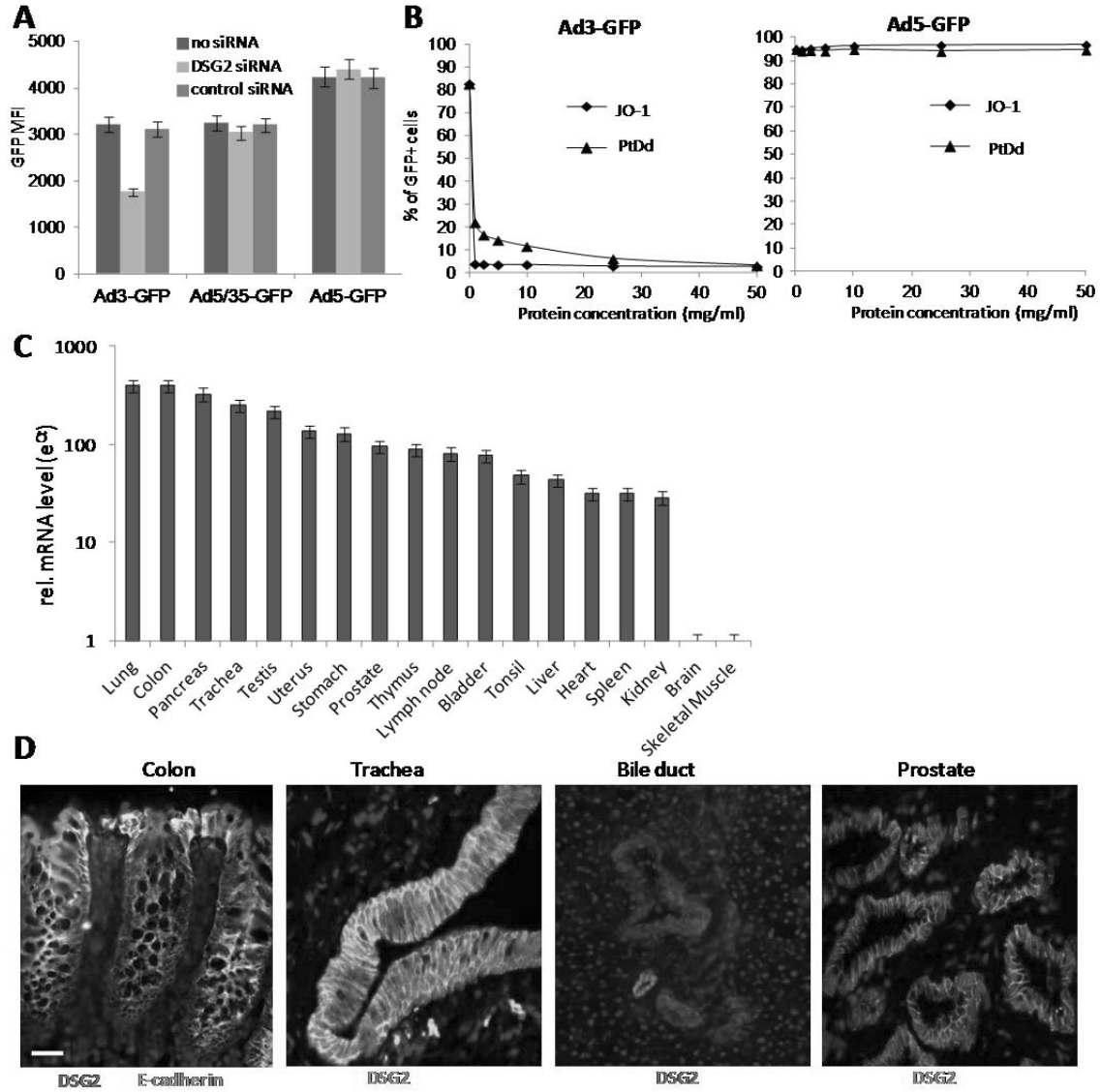


Figure 35. DSG2 expression in tissues of *Macaca fascicularis*. A) DSG2 immunohistochemistry of epithelial tissues. DSG2 appears in brown. **B)** DSG2 immunohistochemistry (left panel) and immunofluorescence analysis (middle and right panels) of tissues with epithelial ducts. The pancreas and skin sections are also stained for E-cadherin to show localization of DSG2 in epithelial junctions. **C)** DSG2 immunohistochemistry of heart tissue. DSG2 is located in intercalated discs. **D)** DSG2 immunohistochemistry of brain tissue. Left panel: choroid plexus. An area of DSG2-positive epithelial cells is labeled by arrows. Right panel: A brain blood vessel reflecting the blood-brain-barrier (see arrow). Representative sections are shown. The scale bars are 20 μm.

4.6.2 **Safety study in Macaca fascicularis**

A preliminary study has been conducted in which two females (~6kg) were injected intravenously with a single dose of JO-1 (0.6mg/kg). This dose corresponds to 2mg/kg in mice based on allometric scaling. The animals were monitored by an independent group of veterinarians and pathologists at the

Washington State Northwest Primate Research Center (WaNPRC) at the University of Washington. A full necropsy was performed at day 3 after JO-1 injection. There was no evidence of changes in health/behavior (note: no evidence of diarrhea), laboratory studies or pathologies (Figs.36 and 37). Histological analysis revealed a mild gastro-enterocolitis (Figs. 38 and 39), which is often observed in monkeys in this center. JO-1 serum clearance and JO-1 biodistribution were similar to those observed in hDSG2 transgenic mice, i.e . JO-1 was found in the GI-tract as well as in macrophages of the liver and lymph nodes (Fig.40). JO-1 was also detected on jejunum sections by immunofluorescene analysis using antibodies against JO-1 (Fig.41). Blood analyses revealed a mild transaminitis at day 1 after injection (Fig.42). This is most likely related to the infusion of the protein preparation, which contained trace amounts of bacterial endotoxin. Consistent with this conclusion, an elevation of serum interferon gamma was also observed (Fig.43). The latter could also be the result of JO-1 uptake by Kupffer cells. Overall, however, blood analyses did not show any critical abnormalities.

A10271

General Distress & Humane Endpoint Scoring Sheet for Nonhuman Primates

Parameter	Degree of parameter	Date:	7/20/12	7/21/12	7/22/12	7/23/12			
		Possible Score	Score	Score	Score	Score	Score	Score	Score
Fever	Normal (37.5-38.5°C)	0	0	0		0			
	Elevated temperature (>38.5-39.5°C)	3							
	High temperature) (>39.5°C)	5							
Posture	Piloerection of body hair	1							
	Decreasing activity, Decreasing normal behaviour, piloerection	2							
	Huddled on camera, active when in room	3							
	Huddled when in room, shaking, toes and hands clenched	5							
Respiration	Normal	0	0	0	0	0			
	Increased or Decreased, mild cough and clear nasal discharge	5							
	Laboured, breathing through mouth; severe cough and severe nasal discharge	10							
Feces + Urine	Normal consistency volume/Soft normal stool	0	0	0	0	0			
	Feces absent or dry or decreased urine output or cloudy urine	2							
	Wet pasty or small very dry stool	2							
	Liquid stool or blood in stool or urine or no urine	10							
Food + water	Normal	0	0	0	0	0			
	Slightly decreased	1							
	Decreased	5							
	Severely decreased, dehydration apparent	10							
Recumbent	No symptoms	0	0	0	0	0			
	Occasionally lies down, huddled, active when people in room	5							
	Lies down, will get up when approached or prompted, uses cage for support	10							
	Lies down, will not get up when approached or prompted	15							
Attitude	Normal	0	0	0	0	0			
	Hyperactive or mildly depressed, responds to treats and toys	1							
	Moderately depressed, response requires prodding, loses interest in treats and toys,	3							
	Severely depressed, no interest in treats, does not respond to human presence	5							
Skin	Flushed appearance to skin	2		Ring	Ring	Ring			
	Visible Rash	5							
	Bleeding	5-10							
Total score*		0-70*	0	1	2	2			

*A veterinarian or veterinary technician will check on the animals daily. * The PI will consult with a veterinarian regarding euthanasia when a score of ~35 is reached.*

Figure 36. Pathology report for animal A10271 after necropsy at day 3 after JO-1 injection

A10272

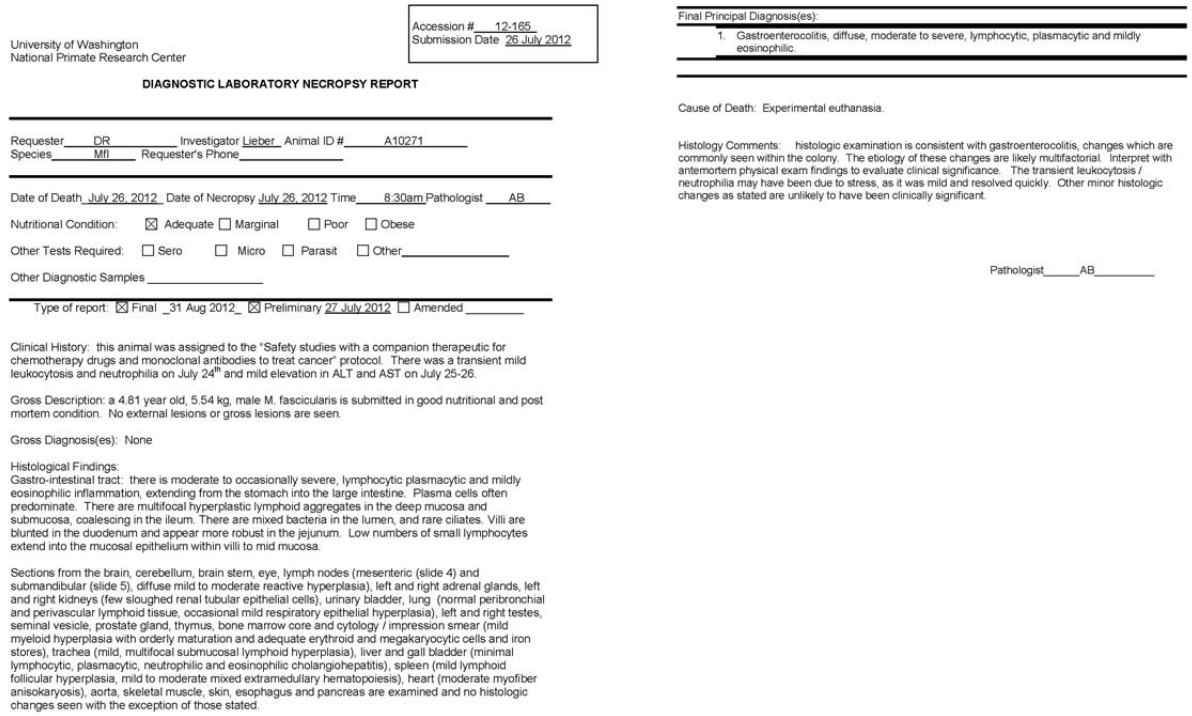
General Distress & Humane Endpoint Scoring Sheet for Nonhuman Primates

Parameter	Degree of parameter	Date:	7/23/12	7/24/12	7/25/12	7/26/12			
		Possible Score	Score	Score	Score	Score	Score	Score	Score
Fever	Normal (37.5-38.5°C)	0	0	0	0	0			
	Elevated temperature (>38.5-39.5°C)	3							
	High temperature) (>39.5°C)	5							
Posture	Piloerection of body hair	1							
	Decreasing activity, Decreasing normal behaviour, piloerection	2							
	Huddled on camera, active when in room	3							
	Huddled when in room, shaking, toes and hands clenched	5							
Respiration	Normal	0	0	0	0	0			
	Increased or Decreased; mild cough and clear nasal discharge	5							
	Laboured, breathing through mouth; severe cough and severe nasal discharge	10							
Feces + Urine	Normal consistency volume/Soft normal stool	0	0	0	0	0			
	Feces absent or dry or decreased urine output or cloudy urine	2							
	Wet pasty or small very dry stool	2							
	Liquid stool or blood in stool or urine or no urine	10							
Food + water	Normal	0	0	0	0	0			
	Slightly decreased	1							
	Decreased	5							
	Severely decreased, dehydration apparent	10							
Recumbent	No symptoms	0	0	0	0	0			
	Occasionally lies down, huddled, active when people in room	5							
	Lies down, will get up when approached or prompted, uses cage for support	10							
	Lies down, will not get up when approached or prompted	15							
Attitude	Normal	0	0	0	0	0			
	Hyperactive or mildly depressed, responds to treats and toys	1							
	Moderately depressed, response requires prodding, loses interest in treats and toys,	3							
	Severely depressed, no interest in treats, does not respond to human presence	5							
Skin	Flushed appearance to skin	2		Ring	Ring	Ring			
	Visible Rash	5							
	Bleeding	5-10							
Total score*		0-70*	0	2	2	2			

*A veterinarian or veterinary technician will check on the animals daily. * The PI will consult with a veterinarian regarding euthanasia when a score of ~35 is reached.*

2

Figure 37. Pathology report for animal A10272 after necropsy at day 3 after JO-1 injection



University of Washington
National Primate Research Center

Accession #___12-165_
Submission Date 26 July 2012

DIAGNOSTIC LABORATORY NECROPSY REPORT

Requester____DR_____ Investigator Lieber_ Animal ID #_____A10271_____
Species_____Mfl____ Requester's Phone_____

Date of Death_July 26, 2012_ Date of Necropsy July 26, 2012 Time____8:30am Pathologist ___AB____

Nutritional Condition: ☒ Adequate ☐ Marginal ☐ Poor ☐ Obese

Other Tests Required: ☐ Sero ☐ Micro ☐ Parasit ☐ Other_____

Other Diagnostic Samples _____

Type of report: ☒ Final _31 Aug 2012_ ☒ Preliminary 27 July 2012 ☐ Amended _____

Clinical History: this animal was assigned to the "Safety studies with a companion therapeutic for chemotherapy drugs and monoclonal antibodies to treat cancer" protocol. There was a transient mild leukocytosis and neutrophilia on July 24th and mild elevation in ALT and AST on July 25-26.

Gross Description: a 4.81 year old, 5.54 kg, male M. fascicularis is submitted in good nutritional and post mortem condition. No external lesions or gross lesions are seen.

Gross Diagnosis(es): None

Histological Findings:
Gastro-intestinal tract: there is moderate to occasionally severe, lymphocytic plasmacytic and mildly eosinophilic inflammation, extending from the stomach into the large intestine. Plasma cells often predominate. There are multifocal hyperplastic lymphoid aggregates in the deep mucosa and submucosa, coalescing in the ileum. There are mixed bacteria in the lumen, and rare ciliates. Villi are blunted in the duodenum and appear more robust in the jejunum. Low numbers of small lymphocytes extend into the mucosal epithelium within villi to mid mucosa.

Sections from the brain, cerebellum, brain stem, eye, lymph nodes (mesenteric (slide 4) and submandibular (slide 5), diffuse mild to moderate reactive hyperplasia), left and right adrenal glands, left and right kidneys (few sloughed renal tubular epithelial cells), urinary bladder, lung (normal peribronchial and perivascular lymphoid tissue, occasional mild respiratory epithelial hyperplasia), left and right testes, seminal vesicle, prostate gland, thymus, bone marrow core and cytology / impression smear (mild myeloid hyperplasia with orderly maturation and adequate erythroid and megakaryocytic cells and iron stores), trachea (mild, multifocal submucosal lymphoid hyperplasia), liver and gall bladder (minimal lymphocytic, plasmacytic, neutrophilic and eosinophilic cholangiohepatitis), spleen (mild lymphoid follicular hyperplasia, mild to moderate mixed extramedullary hematopoiesis), heart (moderate myofiber anisokaryosis), aorta, skeletal muscle, skin, esophagus and pancreas are examined and no histologic changes seen with the exception of those stated.

Final Principal Diagnosis(es):

1. Gastroenterocolitis, diffuse, moderate to severe, lymphocytic, plasmacytic and mildly eosinophilic.

Cause of Death: Experimental euthanasia.

Histology Comments: histologic examination is consistent with gastroenterocolitis, changes which are commonly seen within the colony. The etiology of these changes are likely multifactorial. Interpret with antemortem physical exam findings to evaluate clinical significance. The transient leukocytosis / neutrophilia may have been due to stress, as it was mild and resolved quickly. Other minor histologic changes as stated are unlikely to have been clinically significant.

Pathologist_____AB_____

Figure 38. Diagnostic laboratory necropsy report for animal A10271

University of Washington
National Primate Research Center

Accession # 12-166
Submission Date July 26, 2012

DIAGNOSTIC LABORATORY NECROPSY REPORT

Requester DR Investigator Lieber Animal ID # A10272
Species Mfl Requester's Phone

Date of Death July 26, 2012 Date of Necropsy July 26, 2012 Time 9:30am Pathologist AB

Nutritional Condition: ☒ Adequate ☐ Marginal ☐ Poor ☐ Obese

Other Tests Required: ☐ Sero ☐ Micro ☐ Parasit ☐ Other

Other Diagnostic Samples

Type of report: ☒ Final 31 Aug 2012 ☒ Preliminary July 27, 2012 ☐ Amended

Clinical History: this animal was assigned to the "Safety studies with a companion therapeutic for chemotherapy drugs and monoclonal antibodies to treat cancer" protocol. The hematology data shows leukocytosis and neutrophilia July 23-26, with monocytosis in the 26th, and mild elevation in AST on July 24 and 26.

Gross Description: a 5 year old, 6.96 kg, male M. fascicularis is submitted in good nutritional and post mortem condition. No external lesions are seen. Internal organs are grossly normal.

Gross Diagnosis(es): None

Gross comments: No gross lesions are seen. Microscopic examination is pending.

Histological Findings:
Brain stem: there is multifocal, mild perivascular acute hemorrhage within the brain stem, unassociated with inflammation or gliosis, interpreted to be most consistent with an agonal change.

Lung: there is multifocal, minimal to mild interstitial, mixed and neutrophilic pneumonia with scattered syncytial cells, mild alveolar edema and mild type II pneumocyte hyperplasia. Alveoli in these areas contain small amounts of foamy macrophages or are obscured by collapsed interstitium. Peribronchial and perivascular lymphoid aggregates appear to be normal.

Left and right testes: there are low numbers of multinucleated cells within the seminiferous tubules, along with sloughed tubular cells. Spermatogenesis appear to be progressing in some tubules and is arrested or sparse in others. The epididymis contains dense clusters of spermatids in some tubules and other are sparse.

Seminal vesicles: tubules contain lakes of eosinophilic proteinaceous material and oval basophilic structures (spermatid heads). There are overall low numbers of spermatids.

Prostate gland: There are rare small aggregates of lymphocytes and plasma cells with fewer neutrophils within the interstitial connective tissue. Small lymphocytes occasionally extend into the tubular epithelium. The tubular lumens are predominantly empty or contain small amounts of protein material, cell debris and sloughed epithelial cells.

Spleen: lymphoid follicles appear to be of normal size, with occasional mild hyperplasia. The splenic stroma is congested with abundant extramedullary hematopoiesis, especially of myeloid cells progressing to numerous mature neutrophils.

Bone marrow: there is moderate to occasionally marked myeloid hyperplasia, progressing to mature neutrophils and eosinophils. Erythroid cells appear adequate and are maturing, and adequate megakaryocytes are present. Iron is sparse.

Gastro-intestinal tract: there is diffuse, moderate to occasionally marked lymphocytic, plasmacytic inflammation within the stomach, small and large intestines. Fewer eosinophils and neutrophils are seen. Villi are blunted. There are multifocal hyperplastic lymphoid aggregates and follicles in the deep mucosa to submucosa. Mixed bacteria and ingesta are present in the lumen.

Sections from the cerebrum, cerebellum, pituitary gland, eye, inguinal (slide 5), mesenteric (slide 6) and submandibular (slide 7) lymph nodes (mild to moderate lymphoid follicular hyperplasia with focal tattoo ink (inguinal node)), left and right adrenal glands, left and right kidneys (rare, mild interstitial lymphocytic, plasmacytic nephitis and rare tubular epithelial cell degeneration and luminal proteinaceous material), liver (mild lymphocytic, plasmacytic periportal cholangiohepatitis, mild centrilobular hepatocellular vacuolization), urinary bladder, heart (mild anisokaryosis), thymus, pancreas, trachea, esophagus, aorta, thyroid and parathyroid gland, aorta, skin, skeletal muscle and examined and no abnormalities found with the exception of those minor stated changes described above.

Final Principal Diagnosis(es):

1. Multifocal, minimal to mild interstitial pneumonia, with mild alveolar histiocytosis; lung.
2. Multifocal reduced spermatogenesis; testes.
3. Splenic extramedullary hematopoiesis with myeloid predominance.
4. Myeloid hyperplasia, moderate to marked: bone marrow.
5. Gastroenterocolitis, diffuse, moderate to marked, chronic to mixed, with villous blunting.

Cause of Death: Experimental euthanasia.

Histology Comments: There is evidence of myeloid hyperplasia with the bone marrow (cytology and fixed tissue) and spleen. This may be associated with an inflammatory stimulis and is consistent with the leukocytosis / neutrophilia seen in the hematology data, with WBC count of 25,440 on July 24th. Stress may be playing a role in the leukocytosis (but is not typically associated with myeloid hyperplasia). No infectious agents are seen within the examined tissue sections. There is diffuse gastro-enterocolitis, which may be contributing to an inflammatory response. Testicular maturation may vary in animals of the age range of 3 to 5 years of age, and the variability in spermatogenesis may be at least partially due to age / immaturity.

Pathologist AB

Figure 39. Diagnostic laboratory necropsy report for animal A10271

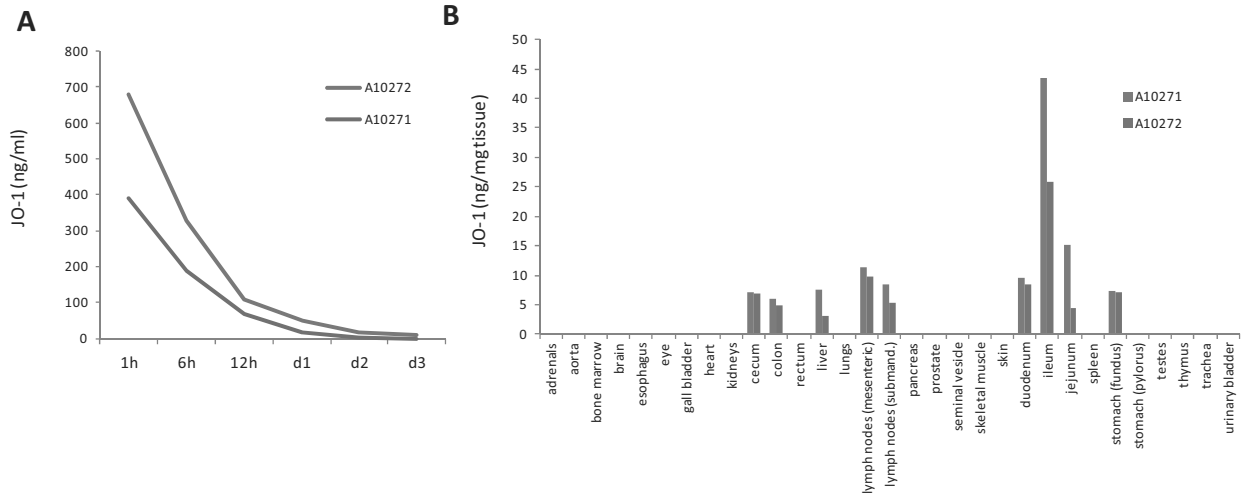


Figure 40. JO-1 levels in macaques afer intravenous injection. A) Serum levels at different time points after JO-1 infusion. **B)** tissue levels of JO-1 at day 3 after infusion. A10271 and A10272 are two male Macaca fasciulcaris (~6kg).

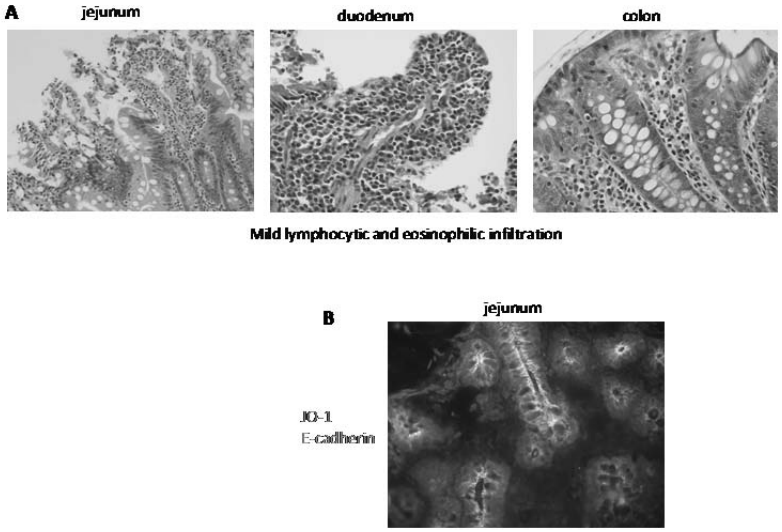


Figure 41. Histology of parts of the gastro-intestinal tract. A) H@E staining demonstrating a mild lymphocytic and eosinophilic infiltration in jejunum, duodenum, and colon. B) Immufluorescence analysis of jejunum sections with anti-JO-1 antibodies and anti-E-cadherin antibodies.

Animal # A10271						**Animal # A10272**				
	pre	Day1	Day2	Day3			pre	Day1	Day2	Day3
PTT	24	24.0	21.0	19		PTT	28.0	28.0	23.0	21.0
Renal/Hepatic Panel						Renal/Hepatic Panel				
Sodium	144.0	143.0		142.0		Sodium	145.0	139.0		141.0
Potassium	3.7	3.9		3.6		Potassium	3.4	3.8		3.5
Chloride	107.0	107.0		104.0		Chloride	107.0	104.0		103.0
Glucose	47.0	79.0		67.0		Glucose	50.0	59.0		63.0
Urea Nitrogen	18.0	26.0		17.0		Urea Nitrogen	14.0	30.0		15.0
Creatinine	0.8	0.8		0.7		Creatinine	0.9	1.0		0.9
Total Protein	6.6	6.6		6.3		Total Protein	7.4	6.8		6.6
Albumin	3.7	3.2		3.2		Albumin	3.5	3.0		2.9
Total Bilirubin	0.4	0.3		0.4		Total Bilirubin	0.2	0.6		0.5
Calcium	9.1	9.0		8.8		Calcium	8.9	8.6		8.8
Phosphate	7.6	4.8		5.9		Phosphate	5.5	5.3		4.5
Total Cholesterol	137.0	106.0		113.0		Total Cholesterol	71.0	91.0		77.0
Alkaline Phosphatase (total)	561.0	477.0		374.0		Alkaline Phosphatase (total)	259.0	275.0		199.0
ALT	47.0	162.0		120.0		ALT	32.0	108.0		89.0
AST	44.0	220.0		68.0		AST	37.0	157.0		77.0
Gamma Glutamyl Transferase	116.0	123.0		109.0		Gamma Glutamyl Transferase	96.0	101.0		77.0
CBC						CBC				
WBC	3.9	14.0	7.8	6.4		WBC	14.5	25.4	17.6	18.3
RBC	6.2	6.2	5.7	5.6		RBC	6.6	6.8	6.2	6.1
Hemoglobin	12.1	12.0	11.0	10.9		Hemoglobin	13.5	13.6	12.4	12.1
Hematocrit	41.0	40.0	38.0	37.0		Hematocrit	45.0	46.0	43.0	41.0
MCV	65.0	64.0	67.0	65.0		MCV	69.0	68.0	49.0	68.0
MCH	19.4	19.2	19.6	19.4		MCH	20.5	19.9	20.0	20.0
MCHC	29.7	29.8	29.4	29.8		MCHC	29.9	29.4	28.8	29.3
RDW-CV	15.7	15.8	16.1	15.7		RDW-CV	13.5	14.8	14.7	14.9
Platelet Count	416.0	314.0	238.0	225.0		Platelet Count	382.0	248.0	190.0	178.0
Neutrophils(%)	45.0	90.0	52.0	49.0		Neutrophils(%)	83.0	89.0	76.0	70.0
Lymphocytes	44.0	10.0	34.0	40.0		Lymphocytes	14.0	5.0	16.0	13.0
Monocytes	9.0	0.0	11.0	9.0		Monocytes	3.0	5.0	8.0	16.0
Eosinophils	2.0	0.0	2.0	2.0		Eosinophils	0.0	1.0	0.0	0.0
Basophil	0.0	0.0	1.0	0.0		Basophil	0.0	0.0	0.0	1.0
Immature Granulocytes	0.0	0.0	0.0	0.0		Immature Granulocytes	0.0	0.0	0.0	0.0
Nucleated RBC			0.0	0		Nucleated RBC			0.0	0
weight	5.7	5.7	5.6	5.5		weight	7.1	7.1	7.2	6.96
temp	37.8	37.8	38.0	38		temp	37.8	37.8	37.9	38

Figure 42. Blood analyses.

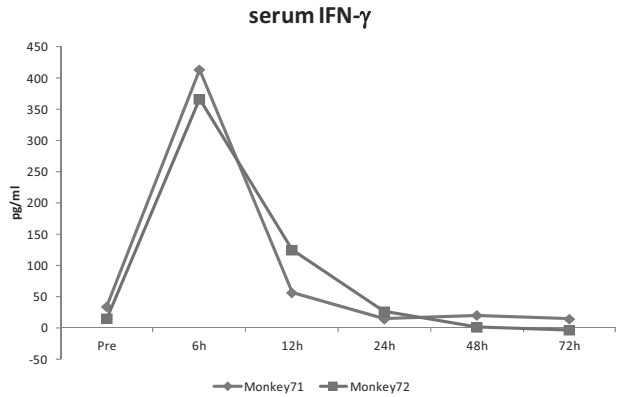


Figure 43. Serum interferon gamma levels at different time points after JO-1 injection into M.fascicularis.

Notably, the PI has just successfully complete an efficacy and safety study with another cancer co-therapeutic protein (Ad35K++) in non-human primates[51] (see Appendix #7).

4.7 Preclinical Results Discussion

4.7.1 Tumor specificity of JO-1 action

We showed that JO-1 co-therapy allows doses of chemotherapeutics to be decreased without compromising anti-tumor effects, and that it provides protective effects to normal tissues resulting in an improved safety profile for chemotherapy. We also demonstrated that JO-1 can overcome resistance of xenograft tumors to a chemotherapy drug. Our data indicate that JO-1 predominantly accumulates in tumors. A number of factors could account for this, including *i)* overexpression of DSG2 on tumor cells, *ii)* better accessibility of DSG2 on tumor cells because of a lack of strict cell polarization, and *iii)* a high degree of vascularization and vascular permeabilty in tumors. Because of its preferential action on epithelial junctions of tumors, JO-1 appears to create a "sink" for chemotherapy drugs, thereby reducing the exposure of normal tissue to these drugs.

4.7.2 JO-1 immunogenicity

JO-1 is a protein derived from Ad3 and therefore, it could potentially be immunogenic. However, this might not be a critical issue if JO-1 is used in combination with chemotherapy, which suppresses immune responses. This prediction is supported by studies with oncolytic adenovirus vectors in which immunosuppression allowed for the repeated application of the vector [52-54]. In addition, we have shown that JO-1 remains active *in vitro* and *in vivo*, even in the presence of anti-JO-1 antibodies generated by the JO-1 vaccination of mice [55]. This may be due to the fact that JO-1 binds to DSG2 with a very high avidity, thus potentially disrupting the complexes between JO-1 and antibodies against JO-1. Notably, JO-1 is a dimer of a trimeric fiber knob, which contributes to the picomolar avidity to DSG2 [3]. We performed repeated injections of JO-1 in an immunocompetent hDSG2 mouse tumor model to test the effect of anti-JO-1 antibodies on the therapeutic efficacy of JO-1. Importantly JO-1 had an enhancing effect on PLD therapy after repeated JO-1 pre-treatment, demonstrating that JO-1 continues to be effective after multiple treatment cycles, even in the presence of detectable antibodies. It can however not be excluded that repeated JO-1 injection into immunocompetent patients results in the development of competing, high-affinity antibodies, or antibodies that may affect the pharmacokinetics of JO-1.

4.7.3 **Risk of metastasis**

JO-1 binding to DSG2 on tumor cells triggers pathways involved in EMT, a process which has been associated with tumor metastasis. However, none of our *in vivo* studies has shown any evidence of increased tumor growth or metastasis after treatment with JO-1. Furthermore, at day 3 after JO-1 injection into mice bearing Her2/neu-positive HCC1954 tumors, there was no significant increase in the percentage of circulating Her2/neu- positive cells in the blood (Fig.20). This is likely due to the fact that tumor metastasis requires more than transient activation of EMT pathways. Detachment from epithelial cancers and migration of tumor cells is only possible after long-term crosstalk between malignant cells and the tumor microenvironment, resulting in changes in the tumor stroma and phenotypic reprogramming of epithelial cells into mesenchymal cells [39]. Transitions between epithelial and mesenchymal cell stages, namely EMT and its reverse mesenchymal-epithelial transition (MET) have been recently accredited important roles in cancer progression, specifically in the induction and maintenance of cancer stem cells [56]. Consequently, we are therefore currently studying the effect of JO-1 on cancer stem cells in xenograft models. To monitor cancer stem cells by non-invasive *in vivo* imaging, we are using primary ovarian cancer cells that express luciferase under Nanog or CD133 promoters.

4.7.4 **Safety of JO-1**

Except for a mild, transient diarrhea, intravenous injection of JO-1 had no critical side effects on other tissues or hematologic parameters in hDSG2 transgenic mice. No significant abnormalities were observed in two macaques treated with JO-1. We speculate that DSG2 in tissues other than the tumor and a small subset of epithelial cells in the GI tract are not accessible to intravenously injected JO-1. The demonstration of safety of JO-1 in combination with PLD in a more comprehensive non-human primate study is required before this therapeutic approach can be tested in humans.

5. PROPOSED TOXICOLOGY STUDY IN NON-HUMAN PRIMATES

For cost and feasibility reasons, we will perform the non-GLP study at Washington State Northwest Primate Research Center (WaNPRC) at the University of Washington (http://www.wanprc.org/). The facility is however not approved for cGLP studies. We will therefore repeat one cohort (highest safe dose of JO-1 plus Doxil, N=10)) at the cGLP approved facility at Bioanalytical Systems, Inc. (BASi) http://www.basinc.com/about/index.html.

5.1 non-GLP study

5.1.1 **Study Objective**

The goal is to generate preclinical safety and pharmacokinetic data for repeated intravenous JO-1 injection (alone and in combination with PLD) in *Macaca fascicularis*. Based on these studies, we will determine the JO-1 dose range to be used in humans.

5.1.2 **Study Design**

All procedures on animals and tissue/blood collection will be performed by the Washington State Northwest Primate Research Center (WaNPRC) at the University of Washington. We will conduct a 6 week toxicology study in *Macaca fascicularis* (N=3) with a JO-1 pilot lot (produced under cGMP-compliant conditions). The first two cohorts will receive intravenous injections of JO-1 alone every other week for 6 weeks. The JO-1 dose for the **first** cohort will be 0.6mg/kg. Based on allometric scaling, this dose corresponds to 2mg/kg in mice, i.e. the dose that was used in all the efficacy studies in mouse models with xenograft tumors and all the studies in hDSG2 transgenic mice. The same dose of JO-1 has

been found to be safe in a three-day safety study with single injection in two *Macaca fascicularis* (see section 4.7.). The **second** cohort will received a "high" dose of 2mg JO-1 per /kg. The **third** cohort will be the highest JO-1 dose found to be safe in cohort 1 and 2 followed by the standard dose of PLD one hour later (every other week for 6 weeks). The standard dose of Doxil used in humans is 50mg/m^2. In a 5kg monkey, this corresponds to a dose of 3.3mg/kg. Notably, a recent study in *Macaca fascicularis* did not show serious toxicity of Doxil following 6 repeated administrations at 2.5 or 4.0 mg/kg (see URL below, "formulation 1" corresponds to Doxil used in patients). Side effects that were observed included scruff, abrasion and skin thickening at injection site, hair loss, moderate increase in RBC, hematocrit, hemoglobin, AST, ALT as well as a moderate decrease in PT and WBC counts.
http://www.ncbi.nlm.nih.gov/pubmed?term=PEGylated%20liposomal%20doxorubicin%20macaques

Table 8: Dosing schedule for non-GLP toxicology study (total amount of JO-1 required: 290mg)

Group #	Dosing	Route	Regiment	Duration	Number
1	JO-1 (0.6 mg/kg)	IV	Every two weeks	6 weeks	3
2	JO-1 (2 mg/kg)	IV	Every two weeks	6 weeks	3
3	JO-1 (highest tolerated dose) + PLD (50 mg/m^2)	IV	Every two weeks	6 weeks	3

5.1.3 **Monitoring**

Physical examinations will be performed daily by a qualified veterinarian. Body weights and EKG will be recorded once prior to treatment and weekly thereafter. Blood samples will be collected from all animals on day 1 of injection at 4 time points – pre-dose, 1, 2, 4, 6, 24 hours post-dose, and then twice per week. Samples will be analyzed for *i)* full blood CBC and chemistry, and *ii)* serum cytokines, *iii)* JO-1, *iv)* anti-JO-1 antibody, and *iv)* (in cohort 3) for PLD concentrations. After 6 weeks, animals will be euthanized and a gross necropsy will be performed by a certified veterinary pathologist. A standard set of tissues (~20/animal) will be collected and examined by a veterinary pathologist. An audited draft of the final report will prepared. JO-1 and PLD concentrations will be measured in all collected tissues by ELISA. Tissue sections will be analyzed by immunohistochemistry for the presence of JO-1 and PLD.

For evaluating toxicity, we will use the following scoring sheet, which is routinely used by the University of Washington Regional Primate Center (Fig.44). The PI will consult with a veterinarian regarding euthanasia when a score of –35 is reached.

Patient ID #

Legend for clinical signs letter designations:

N=normal	**F**=facial	**A**=absent	**N**=nares	**N**=normal	**H**=hyperact.	**N**=normal	**N**=normal	**A**=absent
I=increased	**C**= cervical	**N**=normal	**G**=gums	0-5%	**N**=normal	**P**=picky	**I**=increased	**N**=normal
D=decreased	**O**= other	**I**=increased &	**PS**=puncture	**M**=moderate	**D**=depressed	**A**=anorexic	**D**=decreased	**P**=pasty
L=laboured	(specify)	specify axillary	site	5-9%	**U**=unresponsive			soft/formed
C=coughing		inguinal,other	**Pet**=petechial	**S**=severe	**P**=prostrate			**L**=liquid
			specify site	>9%				

add vit C

Circle one letter in each column.

	Date	**Temp. ^{0}C**	**Respiration**	**Edema**	**Lymph nodes**	**Haemorrhages**	**Hydration**	**Demeanor**	**Appetite**	**Water cons.**	**Feces**
Norm.val.>			40 (30-54)						(350-550 g)	(350-950 ml)	**condition**
Reading>	**Body weight**		**N/ I/ D/ L/ C**	**F/ C /O**	**A/N/I**(specify)	**N/G/PS/Pet.**	**N/ M/ S**	**H/N/D/U/P**	**N/ P/ A**	**N/ I/ D**	**A/N/P/L**

Observation/Analysis

Action/comments:

	Date	**Temp. ^{0}C**	**Respiration**	**Edema**	**Lymph nodes**	**Haemorrhages**	**Hydration**	**Demeanor**	**Appetite**	**Water cons.**	**Feces**
Norm.val.>			40 (30-54)						(350-550 g)	(350-950 ml)	**condition**
Reading	**Body weight**		**N/ I/ D/ L/ C**	**F/ C /O**	**A/N/I(specify)**	**N/G/PS/Pet.**	**N/ M/ S**	**H/N/D/U/P**	**N/ P/ A**	**N/ I/ D**	**A/N/P/L**

Observation/Analysis

Action/comments:

Added physiological data:

NHP: **Urine** excreted daily = 150-550 ml
Heart rate (mean & range): 220 (165-243) /minute
Body temperature: 39 ^{0}C (normal)

Reference: CCAC Guide to the Care and use of Experimental Animals, Vol.1, 2nd edition (fileexcel/animal forms/cliniclaexam…)

Parameter	Degree of parameter	Date:							
		Possible Score	Score	Score	Score	Score	Score	Score	Score
Fever	Normal (37.5-38.5˚C)	0							
	Elevated temperature (>38.5-39.5˚C)	3							
	High temperature) (>39.5˚C)	5							
Posture	Piloerection of body hair	1							
	Decreasing activity, Decreasing normal behaviour, piloerection	2							
	Huddled on camera, active when in room	3							
	Huddled when in room, shaking, toes and hands clenched	5							
Respiration	Normal	0							
	Increased or Decreased; mild cough and clear nasal discharge	5							
	Laboured, breathing through mouth; severe cough and severe nasal discharge	10							
Feces + Urine	Normal consistency volume/Soft normal stool	0							
	Feces absent or dry or decreased urine output or cloudy urine	2							
	Wet pasty or small very dry stool	2							
	Liquid stool or blood in stool or urine or no urine	10							
Food + water	Normal	0							
	Slightly decreased	1							
	Decreased	5							
	Severely decreased, dehydration apparent	10							
Recumbent	No symptoms	0							
	Occasionally lies down, huddled, active when people in room	5							
	Lies down, will get up when approached or prompted, uses cage for support	10							
	Lies down, will not get up when approached or prompted	15							
Attitude	Normal	0							
	Hyperactive or mildly depressed, responds to treats and toys	1							
	Moderately depressed, response requires prodding, loses interest in treats and toys,	3							
	Severely depressed, no interest in treats, does not respond to human presence	5							
Skin	Flushed appearance to skin	2							
	Visible Rash	5							
	Bleeding	5-10							
Total score*		0-70*							

Figure 44. Scoring sheets A) Clinical Examination Record. **B)** General Distress & Humane Endpoint Scoring Sheet for Nonhuman Primates

5.1.4 **Expected Outcomes**

Safety: We expect to find a JO-1 dose that is safe to administer in combination with PLD. If we reach a toxicity score of greater than 30 in more than 1 animal in cohort #1, we will reduce the dose of JO-1 by 50%. Based on these studies, we will determine the JO-1 dose range to be used in humans.

Additional information: We expect to establish the kinetics of JO-1 blood clearance and the development of anti-JO-1 serum antibodies. We will also collect data on Doxil concentrations in tissues.

5.2 GLP study

This study will be conducted in accordance with the US FDA Good Laboratory Practice Regulations. The Test Site will be at Bioanalytical Systems, Inc. (BASi) http://www.basinc.com/about/index.html. The study director will be Roger Hawks. The IACUC protocol for this study was reviewed and approved by the

BASi Animal Care and Use Committee. We propose to test the highest safe dose of JO-1 (from 4.1.) in combination with the standard dose of Doxil. We will have 10 animals in one cohort.
Physical examinations will be performed daily by a qualified veterinarian on all study animals prior to initiation and on all animals during the study until their respective necropsies. Body weights and EKG will be recorded once prior to treatment and weekly thereafter. Blood samples will be collected from all animals on day 1 of injection at 4 time points – pre-dose, 1, 2, 4, 6, 24 hours post-dose, and then twice per week. The samples will be centrifuged, the plasma separated, and then frozen and shipped to the PI's laboratory for analysis of: *i)* full blood CBC, coagulation parameters, and chemistry, and *ii)* serum cytokines, JO-1, anti-JO-1 antibody concentrations. After 12 weeks, animals will be euthanized with sodium pentobarbital, exsanguinated, and a gross necropsy will be performed by a certified veterinary pathologist at BASi. A standard set of tissues (~ 66/animal) will be collected and preserved in 10% formalin. All collected tissues will be trimmed, embedded in paraffin, sectioned, and then slides prepared and examined by a veterinary pathologist. An audited draft of the final report will be available 6 weeks following the final necropsy. Specific attention will be given to GI-tract and cardiac toxicities, based on previous murine toxicology studies of JO-1, which showed mild, transient diarrhea, and the well-known cardiac toxicity associated with anthracyclines, such as PLD.

Table 9: GLP toxicology study design (total amount of JO-1 required: 420mg)

Group #	Dosing	Route	Regiment	Duration	Number
1	JO-1 (highest tolerated dose) + PLD (50 mg/m^2)	IV	Every two weeks	12 weeks	10 females

5.3 Statistical power calculations

For the toxicology studies, we selected a group size of 10 animals. We arrived at this number by setting a large effect size, which was based on our previous NHP studies, and a desired power of 0.8 at a significance level of 0.05. Using the pwr package of the R statistical software bundle to calculate the number of animals needed, we arrived at 9.6. Therefore, 10 animals will be in each treatment group.

6. PREVIOUS HUMAN EXPERIENCE

There is no previous human experience with JO-1. JO-1 contains the fiber knob domain of adenovirus serotype 3 and binds to DSG2 in a way similar to Ad3. This implies that studies performed with Ad3 or Ad3 fiber knob containing Ads are relevant for predicting potential side effects of JO-1 in a clinical trial. For more than a decade, Ad5 vectors that possess Ad3 fibers (Ad5/3), which also infect cells through hDSG2 [3], have been used for cancer therapy in animal models and humans with a very good safety profile [57,58]. Last year, we have performed as study with an oncolytic vectors based on Ad3 [59]. In this study, twenty-five patients with chemotherapy refractory cancer were treated with a fully serotype 3-based oncolytic adenovirus Ad3-hTERT-E1A. The only grade 3 adverse reactions observed were self-limiting cytopenias. Neutralizing antibodies against Ad3 increased in all patients. Signs of possible efficacy were seen in 11/15 (73%) patients evaluable for tumor markers, four of which were treated only intravenously. Particularly promising results were seen in breast cancer patients and especially those receiving concomitant trastuzumab. Taken together, Ad3-hTERT-E1A seems safe for further clinical testing or development of armed versions.

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8. APPENDIX A – PDFS OF PUBLICATIONS

1. Wang, H., Li, ZY., Liu, Y., Persson, J., Beyer, I., Möller, T., Koyuncu, D., Drescher, M.R., Strauss, R., Zhang, XB., Wahl, JK, Urban, N., Drescher, C., Hemminki, A., Fender, P., Lieber, A. (2011) Desmoglein 2 is a receptor for adenovirus serotypes 3, 7, 11, and 14.
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Molecular Therapy.;21(2):291-9.

8. Beyer, I., van Rensburg, R., Lieber, A. 2013 Overcoming physical barriers in cancer therapy
Landes Bioscience, accepted

FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH

PIND 117820

MEETING REQUEST-
WRITTEN RESPONSES

Andre Lieber, MD, PhD
Professor
Department of Medicine
University of Washington
Box 357720
Seattle, WA 98195

Dear Dr. Lieber:

Please refer to your Pre-Investigational New Drug Application (PIND) file for JO-1 tight junction opener with PEGylated liposomal doxorubicin, Doxil®.

We also refer to your submission dated February 14, 2013, containing a pre-IND meeting request. The purpose of the requested meeting was to discuss the planned phase 1 clinical trial on JO-1 junction opener with PEGylated liposomal doxorubicin, Doxil® in patients with progressive, persistent or recurrent ovarian/fallopian tube cancer, who have previously received standard therapies.

We have determined that written responses to your questions would be the most appropriate means for responding to your Pre-IND questions. Therefore, the teleconference scheduled for May 21, 2013, at 2:00 PM EST will be cancelled.

The enclosed document constitutes our written responses based on the information provided in your background package and on the questions, Ms. Christine Poulin sent us via email on your behalf on April 12, 2013.

If you have any questions, call Rajesh Venugopal, Regulatory Project Manager at (301) 796-4730.

Sincerely,

{See appended electronic signature page}

Patricia Cortazar, MD
Clinical Team Leader
Division of Oncology Products 1
Office of Hematology and Oncology Products
Center for Drug Evaluation and Research

Enclosure:
Written Responses

WRITTEN RESPONSES

Meeting Type:	Type B
Meeting Category:	Pre-IND
Application Number:	PIND 117820
Product Name:	JO-1 tight junction opener with PEGylated liposomal doxorubicin, Doxil®
Indication:	Progressive, persistent or recurrent ovarian/fallopian tube Cancer, who have failed standard therapies
Sponsor/Applicant Name:	Andre Lieber, MD, PhD
Regulatory Pathway:	505(b)(1)

1.0 BACKGROUND

Andre Lieber, MD, from University of Washington, requested a pre-IND meeting to discuss the development plan for a "Junction Opener-1" (JO-1) biotherapeutic protein in combination with Doxil for the treatment of patients with recurrent or persistent epithelial ovarian, fallopian tube, or primary peritoneal cancer.

JO-1 is a small recombinant protein that targets desmoglein-2 (DSG-2) in intercellular junctions. JO-1 has a dimerization domain, a linker domain and a DSG2 binding sequence derived from adenovirus type 3. As stated by the sponsor, preclinical studies demonstrate JO-1 binds to tumor tight junctions and open them which may enhance the entry of cancer therapeutics into tumors. There is no human experience with JO-1. The Sponsor cited safe human use of adenovirus-based vectors containing Ad3, from which JO-1 is derived. The Sponsor conducted a study with an oncolytic vectors based on Ad3 in 25 patients with refractory tumors. Grade 3 adverse events were limited to myelosuppression and elevated neutralizing antibodies against Ad3.

The sponsor is planning to conduct a Phase 1 first in human study, to evaluate the safety of JO-1 in combination with PEGylated Lipsomal Doxorubicin (PLD, Doxil) in patients with progressive, persistent, or recurrent ovarian/fallopian tube cancer, who have disease progression after treatment with standard therapies.

2.0 DISCUSSION

Questions and Responses

Clinical

1. Currently available data suggest that single agent JO-1 therapy is unlikely to have a significant therapeutic effect. Our hypothesis, supported by our pre-clinical data, is that JO-1 treatment will enhance the effectiveness of PLD therapy without increasing toxicity. Further compared to single agent JO-1, JO-1/PLD combination therapy is significantly less likely to lead to development of anti-JO-1 antibodies that could impact therapy efficacy. As both the risks and benefits for study patients need to be considered, we elected to design our trial to include JO-1/PLD combination therapy without single agent

JO-1 treatment. It is also notable that this study is sponsored by the FHCRC Ovarian Cancer SPORE with a relatively small budget.

Are there concerns at the Agency regarding this trial design?

FDA Response:
This question is premature. You have not provided us with enough information from your pre-clinical studies to determine the potential concerns with your future clinical study. The proposed eligibility criteria and safety monitoring plan may change depending on the results of your pre-clinical studies. We will review the protocol and provide comments at the time of IND submission.

What is the rationale for treating patients up to 4 cycles of JO-1 and Doxil? Typically studies in the metastatic population plan to treat until disease progression or unacceptable toxicity.

The label for Doxil contains a black box warning regarding serious and sometimes fatal allergic/anaphylactoid infusion reactions. Therefore, particular care should be taken to monitor for these reactions and, as per the Doxil label, medications and emergency equipment to treat such reactions should be available for immediate use.

Non-Clinical

2. The funds that we currently have available for the pre-clinical development of JO-1 are not sufficient to conduct a comprehensive GLP toxicology study at a for-profit CRO such as BASi.

   Can the data from the non-GLP studies conducted at the Washington State Northwest Primate Research Center (WaNPRC)/University of Washington be used to supplement smaller GLP safety studies at BASi?

   FDA Response:
   No. The proposed nonclinical studies are not adequate to support the proposed clinical trial.

   As described in the ICH S9 Guidance for Industry: Nonclinical Evaluation for Anticancer Pharmaceuticals [http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM085389.pdf], general toxicology studies to support a first-in-human clinical trial in patients with advanced cancer should be conducted according to Good Laboratory Practices (21 CFR part 58) in one rodent and one non-rodent species with the route of administration to be used in the clinical trial. An approximation of the clinical schedule should be evaluated in the toxicology studies. See the FDA Redbook 2000 [http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInfo

rmation/IngredientsAdditivesGRASPackaging/ucm2006826.htm] for general information on the design and conduct of general toxicology studies.

Provide an adequate justification in your IND submission for the species used for toxicology studies with JO-1. For additional information on species selection for biotechnology products, including a justification for using a single species, refer to ICH S6 Guidance for Industry: S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals [http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM074957.pdf] and to ICH S6 Addendum to Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals. The justification for species selection provided in the briefing package lacked the supportive data and/or detail (e.g., comparative binding, functional activity, target sequence homology in Human and Non-Human Species).

As one of the objectives of your proposed clinical trial is to determine the maximum tolerated dose and dose limiting toxicities of JO-1 in combination with Doxil, the nonclinical toxicology studies should adequately characterize the toxicities associated with JO-1. Your IND should include an assessment of the potential to recover from toxicities. Including a terminal nondosing period of an adequate duration in toxicity studies is warranted if there is severe toxicity and recovery cannot be predicted by scientific assessment. Please note that in the absence of a recovery groups, you may have an inadequate assessment of the reversibility of toxicities, which could result in your IND being placed on clinical hold.

In addition, we have the following recommendations:

- **Adequate single-dose toxicology studies with appropriate dose levels of JO-1 may support your proposed clinical trial administration schedule of once every 4 weeks.**
- **For non-rodent species, we generally recommend that 4 animals/group be used for main study groups, and 2 animals/group for recovery groups.**
- **Include an assessment of toxicokinetics in the general toxicology studies.**
- **In your IND submission, provide an adequate starting dose justification for the proposed clinical trial based on the nonclinical studies.**

The JO-1 drug substance used in the nonclinical studies should be well characterized and adequately represent the active substance to be used in clinical trials. In your IND submission, provide relevant CMC information from the lot(s) used in nonclinical toxicology studies to allow us to compare to the lot(s) to be used in the proposed clinical trial.

See ICH S9 for recommendations on combination of pharmaceuticals to be used in patients with advanced cancer. We recommend that you conduct adequate nonclinical toxicology studies, as described above, with JO-1 alone. In addition,

based on the proposed mechanism of action of JO-1, we recommend that you include an arm of JO-1 in combination with Doxil in your GLP toxicology studies.

Immunogenicity

3. We were not able to generate JO-1 (with >95% purity) by methods other than His-tag/NTA agarose affinity purification.

   Are there concerns at the Agency regarding the use of a 6× histidinyl tagged therapeutic protein?

FDA Response:

Comments regarding antibody monitoring:

1. **A thorough description of the anti-JO-1 binding antibody assay should be provided in your IND submission.**

2. **You have found that there is some expression of the DSG2 receptor on Intestinal Epithelial cells (IEC), which can bind JO-1. You hypothesize that this JO-1 binding to IEC can trigger opening of epithelial junctions, increase intestinal permeability, and allow an influx of intestinal bacteria or bacterial antigens, which in turn causes a TLR mediated expression of inflammatory cytokines and subsequent intestinal injury. Stimulation of IECs through TLR can also induce IgA expression (Abreu (2010) Nature Reviews in Immunology 10, pp. 131-143;Sutherland and Fagarasan (2012) Curr. Opinion Imm. 24, pp. 261-268). Therefore, the FDA recommends that you use your binding antibody assay to detect IgA, as well as IgG and IgM.**

3. **Your IND submission should include a risk assessment for potential increased immunogenicity due to the 6x His tag on the N terminal of the JO-1 protein.**

4. **Doxil consists of doxorubicin HCL encapsulated in STEALTH liposomes coated with an outer layer of methoxypolyethylene glycol (MPEG). Because anti-PEG antibodies have been related to clinical adverse reactions for some pegylated drugs, you should develop an assay to assess the presence of pre-existing and treatment induced anti-PEG antibodies in serum samples.**

5. **For patients who become seropositive, binding antibodies should be monitored until they return to baseline.**

6. **Validated assays for binding antibodies and neutralizing antibodies should be in place by the time Phase III studies are performed.**

Comment regarding assessment of anti-tumor and anti-JO-1 T cell activity:

1. **The FDA notes that analyzing for subsets of reactive T-cells may be helpful in understanding anti-tumor responses resulting from JO-1 treatment.**

Chemistry, Manufacturing and Control

4. *Are the proposed release test methods and specifications for the JO-1 therapeutic acceptable to FDA?*

FDA Response:
No. The proposed test methods and specifications for release testing of JO-1 drug product (DP) are not sufficient to determine whether the DP is safe to use in the phase 1 clinical study. The following comments and recommendations should help clarifying our position regarding release test methods and specifications:

1. **The Particulate Matter in injection test (USP<788>) is not part of your proposed DP release test methods. Protein particles (visible and sub-visible) can be generated from protein alone or from heterogeneous nucleation on foreign micro- and nanoparticles. The levels and sizes of protein particles present in a given product can be changed by many factors relevant to commercial production of therapeutic proteins. Particles of therapeutic proteins formed by adsorption of protein molecules onto foreign micro- and nanoparticles might be particularly prone to cause immunogenicity (Xiang et al. 2006. Methods 40:1-9). Unless appropriate quality controls are in place for sub-visible particles, a product that was safe and effective in clinical trials may unexpectedly cause adverse events in patients after commercialization. Thus, the presence of particulate matter in any injectable solution is a potential safety issue. Please add the USP<788> test for Particulate Matter to your list of DP release test methods.**

2. **Measurements of host cell protein (HCP) impurities should be added to your proposed DP release test methods along with an appropriate acceptance criterion for this test method. A summary description of the assay and the source (in-house or commercial) of the antiserum used for detection of HCPs should be provided. The anti-HCP antiserum needs to be qualified for its ability to detect potential HCP impurities. This assessment should include 2D SDS-PAGE gels of the range of HCPs detected by a sensitive protein stain, such as silver stain, compared to the range detected by western blot analysis using the antiserum employed in the assay. It is possible to use a similarly sensitive and discriminating assay in lieu of the 2D SDS-PAGE assay. If an alternative pathway is pursued, consultation with the Agency is recommended. These data should be used to determine the approximate percent of potential HCP impurities that are recognized by the HCP antiserum. It is the Agency's experience that analysis of HCP coverage by a 1-dimensional SDS-PAGE gel method is not sufficiently informative for this purpose.**

3. **Include additional assays that measure potency, protein aggregates (e.g. size-exclusion-HPLC) and purity [e.g. RP-HPLC, ion-exchange HPLC, hydrophobic interaction chromatography (HIC)] as DP release tests. Appropriate acceptance**

criteria for these tests should be established and have upper and lower limits, where applicable.

4. **The proposed SDS-PAGE (reduced) DP release test should also be performed under non-reduced conditions using coomassie and silver stains (or stains of comparable sensitivity).**

5. **Your proposed release acceptance criterion for DP protein content should include an upper limit.**

The following product quality comments and recommendations should be helpful when preparing an IND submission:

1. **All raw materials used in the manufacturing of JO-1 should be fully qualified and depending on the complexity of the raw material and adequacy of the certificate of analysis supplied by the vendor(s), supplemental in-house testing may be required.**

2. **Information on the generation and characterization of Master Cell Bank (MCB) and Working Cell Bank (WCB) should be included in the IND submission. We recommend that both MCB and WCB be characterized for microbial identification, purity (plasmid DNA and bacterial host cells) and stability (retention of the recombinant construct and selectable markers in the host cells). For additional information on cell bank systems, refer to ICH guidances Q5B and Q5D.**

3. **You did not provide any information on the characterization of the drug substance (DS). You should extensively characterize the DS using a large battery of physicochemical tests. Additionally, process-related and product-related impurities if present in high levels should be characterized and assessed for product safety. If this cannot be accomplished, the impurities levels should then be controlled to ensure product lot-to-lot consistency. [See ICH Q3A (Impurities in new drug-substance), Q3B (Impurities in new drug product) and Q3C (Impurities: Residual Solvents)].**

4. **You did not provide any information on the test methods used for release testing the DS. Please be advised that according to CFR 312.23 an IND submission must contain, "the acceptable limits and analytical methods used to assure the identity, strength, quality, and purity of the drug substance."**

    a. **Identity test methods should uniquely identify your product (e.g. peptide mapping for DS and Western blot analysis or ELISA with a specific antibody for DP).**

b. Strength test methods should be sensitive, quantitative and reproducible in determining the protein concentration of the DS and DP (e.g. UV or any other method of equivalent sensitivity).

c. Purity test methods should also be sensitive, quantitative and reproducible. Purity test methods include, but not limited to, reverse-phase HPLC, size-exclusion HPLC, ion-exchange HPLC, hydrophobic interaction chromatography, isoelectric focusing (IEF) or capillary IEF, SDS-PAGE [reduced and non-reduced, coomassie and silver stains (or stains of comparable sensitivity)] and capillary electrophoresis.

d. Potency test methods should be as relevant as possible to the product's proposed biological mechanism of action (i.e., should reflect the proposed clinical function).

e. Safety test methods include pH, appearance, DNA contamination, host cell protein contamination, bioburden, endotoxin and aggregates.

f. The protein JO-1 contains two cysteine residues (C-81 and C-193); should these cysteines be critical for product function, the presence of free sulfhydryl should be controlled. This would require developing a DS release assay for free sulfhydryl measurements and setting appropriate limits for this test.

g. Acceptance criteria for each DS release test method should be established and justified based on data obtained from: (1) the quality attributes of lots used in preclinical and/or clinical studies and of lots used for demonstrating manufacturing consistency; and (2) stability studies. Please refer to ICH Q6B for guidance. Moreover, specifications should be quantitative where applicable with upper and lower limits. For early clinical studies, specifications may be broad, but should be representative of the current manufacturing capabilities.

5. You should produce enough material to complete the phase 1 trial. In addition, we recommend that you provide information on the manufacturing process(es) used for the production of the non-clinical and clinical JO-1 lots, highlighting differences in manufacturing if any. Should there be differences in the manufacturing process of the non-clinical and clinical lots, the results of a side-by-side physicochemical comparison of JO-1 lots used in the non-clinical studies and those to be used in the proposed clinical studies must be provided along with qualitative and quantitative supporting data.

PREA REQUIREMENTS

Under the Pediatric Research Equity Act (PREA) (21 U.S.C. 355c), all applications for new active ingredients, new indications, new dosage forms, new dosing regimens, or new routes of administration are required to contain an assessment of the safety and effectiveness of the product for the claimed indication(s) in pediatric patients unless this requirement is waived, deferred, or inapplicable. Further, under the Food and Drug Administration Safety and Innovaton ACT (FDASIA), sponsors must submit a Pediatric Study Plan (PSP) within 60 days of an End-of-Phase 2 (EOP2) meeting held on or after November 6, 2012.

Because none of the criteria apply to your application at this time, you are exempt from these requirements/Because this drug product for this indication has an orphan drug designation, you are exempt from these requirements.

DATA STANDARDS FOR STUDIES

CDER strongly encourages IND sponsors to consider the implementation and use of data standards for the submission of applications for investigational new drugs and product registration. Such implementation should occur as early as possible in the product development lifecycle, so that data standards are accounted for in the design, conduct, and analysis of clinical and nonclinical studies. CDER has produced a web page that provides specifications for sponsors regarding implementation and submission of clinical and nonclinical study data in a standardized format. This web page will be updated regularly to reflect CDER's growing experience in order to meet the needs of its reviewers. The web page may be found at:
http://www.fda.gov/Drugs/DevelopmentApprovalProcess/FormsSubmissionRequirements/ElectronicSubmissions/ucm248635.htm

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/s/

PATRICIA CORTAZAR
05/20/2013

RESOURCES

Follow the 398 application instructions in Part I, 4.7 Resources.

Translational and Outcomes Research Group
The Fred Hutchinson Cancer Research Center
Seattle, WA

The Fred Hutchinson Cancer Research Center (FHCRC) is an independent, freestanding, nonprofit corporation in the state of Washington. It is organized into seven divisions: the five scientific divisions of Basic Sciences, Clinical Research, Public Health Sciences, Human Biology and Vaccine and Infectious Disease; and the divisions of Administration and Development.

The Division of Public Health Sciences (PHS), the largest of the Hutchinson Center's five scientific Divisions, is composed of five programs – Biostatistics and Biomathematics, Cancer Biology, Cancer Prevention, Computational Biology, Epidemiology and Translational Research. The Division employs over 700 individuals, including 130 member-track and joint faculty investigators assisted by 55 doctoral level staff scientists. Division faculty are supported by highly skilled technical staff, including 37 technical developers, 51 master level statisticians, 53 post-doctoral fellows and 58 pre-doctoral graduate students. The PHS research portfolio consists of over 360 grants and contracts supported by $70 million in annual direct funding. The Translational and Outcomes Research Group is a research program within Translational Research Program.

Laboratory:

Translational and Outcomes Research Group occupies 2 modules of laboratory space in the Robert M. Arnold Public Health Sciences Building on FHCRC's South Lake Union Campus to conduct biomarker validation and high throughput screening. The laboratory is equipped with periphery equipment such as computers and printers. Existing laboratory equipment also includes two BioRad BioPlex machines, a Perkin Elmer Janus robot for automated plate handling, a Biomek fluorescent plate reader, 3 standing incubators and 2 shaking incubators to support yeast and bacterial culture for scFv production and selection, 2 Fisherbrand microcentrifuges, 2 water baths, 3 dry heaters, 4 vortexes, a sonicator, a pH meter, 2 rotators, 2 stirrers, DNA and protein electrophoresis equipment, a bead sorting magnet, one -80°C freezer,2 -20°C freezers and 3 +4°C refrigerators.

The TOR group also uses its laboratory space to conduct study specific specimen processing. Existing laboratory equipment to support this work includes a Fisherbrand microcentrifuge, vortex, Statspin cytocentrifuge, vacuum manifold, and a rotator. A refrigerator/freezer and a dry ice container are located in the lab space for short term specimen storage.

In addition, the TOR group has access to equipment that other FHCRC investigators are willing to share. These include a Beckman benchtop centrifuge, a tissue culture hood and CO2 incubators, a Spectramax ELISA plate reader, a Qiagen TissueLyser for high throughput disruption of tissue samples leading to RNA extraction, an ABI 9700HT real time PCR machine, well-calibrated balances, an Olympus BX40 compound light microscope, and a digital camera for capturing IHC images.

TOR staff also occupy 840 square feet of clinical office space leased from the Marsha Rivkin Center for Ovarian Cancer Research located on the 7th floor of the Heath Building on the Swedish Medical Center campus. This space includes a lab area that contains a small benchtop centrifuge and a Fisherbrand microcentrifuge for separating freshly collected whole blood into its components. There is a dry ice container, a liquid nitrogen dewar and a clinical-grade refrigerator for the short-term storage of specimens and reagents. Another room houses the Abbott ARCHITECT i1000 instrument which is used to conduct FDA-approved clinical biomarker assays, including CA125 and HE4 measurement.

Clinical:

Two dedicated screening rooms are available for use by TOR staff at the Marsha Rivkin Center for Ovarian Cancer Research. They are each equipped with cabinets containing blood collection supplies, chairs for blood collection, and a sink. There is also a designated space available to TOR staff to meet with potential and enrolled participants, and a waiting area with an information table.

Animal: Not applicable

Computer:

FHCRC Information Technology Services (Center IT)

Center IT is staffed with 87 FTE (including Scientific Computing and security staff) and provides support for a complex heterogeneous computing environment. Center IT manages the FHCRC Storage Area Network (SAN) which consists of a 3PAR storage backend and is sharing data via a highly available NetApp storage gateway and 2 high throughput commodity storage gateways to allow maximum collaboration of Windows, Linux and Macintosh computer users. The total networked storage capacity is currently 600 TeraByte. The current system is expected to grow to 1.2 PetaByte by 2013. Data protection is implemented by DataDomain appliances in conjunction with Commvault Simpana backup software and IBM Tivoli Storage Manager.

In order to accommodate the growing demand for computer resources and mitigate the power demands of physical systems, many of the server services are now being provided by virtual systems using the advanced VMWare ESX technology. The virtual systems have the added protection of being recoverable through snapshots which are taken and stored on a daily basis. The ESX vSphere Environment is configured as self-service Enterprise cloud and currently hosts more than 700 virtual machines.

The network infrastructure consists of multiple Local Area Networks (LANs) connected to the Fred Hutchinson Network. Intel personal computers, Macintosh systems, and LINUX/UNIX workstations are connected to servers running different operating systems. The FHCRC network is connected to the Internet through the Pacific Northwest GigaPop Network (PNWGP) with 1 Gbit/s. The FHCRC Network is protected by redundant firewalls and a redundant intrusion prevention system. There are over 3,000 local workstations, printers, and servers connected to the FHCRC Network. FHCRC supports both UNIX/IMAP and Microsoft Exchange for e-mail services. Fax and photocopier machines are also available throughout the Center. The FHCRC computing environment serves the needs of the proposed project well. Not only does it provide a mechanism for investigators to collaborate with colleagues within the project and within the Center, but also through the PNWGP Network with Consortium partners and through the Internet with institutions around the world. Center IT also provides support for over 60 applications that are made available to the entire Center including an enterprise level SharePoint Collaboration platform.

FHCRC Scientific Computing (Center IT)

The Scientific Computing group is staffed with 5 FTE and provides the following services to FHCRC research groups and Shared Resources:

- High performance computing (2 HPC clusters and large memory machines)
- General Linux/Unix support (Linux Desktop Support, managing applications in departmental Linux servers)
- Software development support (SCM/Subversion source code management, code evaluation for R, C, Python, shell scripting support, software packaging, performance evaluation)
- HPC and Linux/Unix training
- File and data management assistance/data archiving

Specifically, there are three large computing resources available for FHCRC researchers:

- The 'Hyrax' cluster is currently equipped with 270 compute nodes with more than 2700 cores and almost 9TB of main memory (RAM) and is connected to a high performance NetApp storage array and the Enterprise Storage Area Network. It consists of 4 Intel Xeon (X5650) nodes, each with 12 cores/128 GB RAM for a total of 48 cores/512 GB RAM (Head- and development nodes); 49 Intel Xeon (E5345) nodes, each with 8 cores/8 GB RAM for a total of 392 cores/392 GB RAM; 90 AMD “Shanghai” nodes,

each with 8 cores/32 GB RAM for a total of 720 cores/2880 GB RAM; 48 AMD “Istanbul” nodes, each with 12 cores/32 GB RAM for a total of 576 cores/1536 GB RAM; and 85 Intel Xeon (X5650) nodes, each with 12 cores/48 GB RAM for a total of 1020 cores/4080 GB RAM.

- The 'Mercury' cluster is currently equipped with 115 compute nodes with more than 800 cores and more than 3TB of main memory (RAM) and connected to the Enterprise Storage Area Network. It consists of 55 AMD Opteron 2.6 Ghz nodes, each with 2 cores/4 GB RAM for a total of 110 cores/220 GB RAMl and 60 Intel Xeon (X5650) nodes, each with 12 cores/48 GB RAM for a total of 720 cores/2880 GB RAM.
- The 'Sprawl' shared memory system is an IBM 3950 with eight 8 core Intex 7560 processors (64 cores total) and 512GB RAM. The system is connected to a low latency high performance IBM storage array.

Each cluster node possesses a local disk drive which can be used for non-shared temporary data. All of the cluster nodes are 64-bit Linux systems running the current version of Linux operating system. A variety of standard software is installed on the head nodes and on each work node. This includes the current version of R which is updated on a monthly basis as well as the OpenMPI and MPICH parallel processing frameworks.

Security Office (Center IT)
The Security Office is staffed with 4 FTE. The Office maintains a Tipping Point Intrusion prevention system and a clustered Nokia high performance firewall.

PHS Divisional Network Support
The PHS Division is staffed with 4 FTE and provides services that are specific to programs under the PHS umbrella that would otherwise be duplicated by each research group. These include computer-training labs, divisional-shared presentation equipment, and development servers that are made available to projects for prototyping and testing of new applications (apart from production networks).

The network allows PC's, Macintosh, and LINUX workstations continuous 24-hour access to resources. All physical servers have redundant power and network connections, and use RAID 5 to insure protection against drive failure. These virtual systems are supported by multiple hosts and have a high degree of redundancy. Physical servers are backed-up daily through Center-supported tape backup system.

The main PC operating system is Microsoft Windows 7 Professional. The primary application suite used on all computers is Microsoft Office 2010 Professional Enterprise Edition. Remote users can access data servers through the Internet by using a secured Virtual Private Network tunnel. Statistical software used in the program includes R, SAS, SPSS, STATA, SPLUS and MATLAB. Database applications supported in the program include Microsoft SQL Server, Oracle, Sybase, Access and FoxPro.

Special survey research support for computer-assisted telephone interviewing, automated open-ended coding, data entry software and optical scanning is available from the Consortium’s Collaborative Data Services shared resource. The CDS Creative Services unit provides support in the preparation of high quality materials utilizing a variety of graphics packages on both PC and Macintosh platforms. HP Color LaserJet and Epson Stylus Pro printers are available for high quality and large format printing production.

Translational and Outcomes Research (TOR) Network - Specimen and participant tracking systems run on Windows 2003, IIS6, custom code, and FoxPro in a highly available virtual cluster. Internet access to select tracking systems are available and secured using SSL, NTLMv2, and locked down to specific IP addresses. Remote access to TOR systems is available via RDP over SSL, and VPN over SSL.

Office:
TOR office space and furnishings are located on the second floor of the Arnold Public Health Sciences building on the FHCRC South Lake Union campus. Individual offices for scientists, research associates, research support staff, and network support are provided. State of the art conference rooms are also available throughout the building. In addition, projects in the PHS Division can make use of specialized spaces for interviewing, data collection, and medical examination functions.

TOR also occupies office space and furnishings located at the Marsha Rivkin Center for Ovarian Cancer Research the 7th floor of the Heath Building on the Swedish Medical Center campus. TOR occupies 840 square feet of office space with four separate offices (one office with two workstations) and one work stations in the reception area, each equipped with computer workstations, telephones, and secured storage and file cabinets.

Other: Not Applicable

Additional Shared Resources at the FHCRC include a library, core facilities for DNA sequencing, DNA microarrays/expression profiling, computational biology including cluster computing, electron microscopy, and proteomics. Shared resources includes a Biostatistics program for correlation and collaborative statistical support; and Image Analysis program which includes a Phosphor Imager, and Delta Vision microscope. Glassware washing is centralized. All FHCRC shared resources operate on a charge back basis.

MAJOR EQUIPMENT:

As noted above: 1 Abbott ARCHITECT i1000, 2 BioRad BioPlex machines, a Perkin Elmer Janus robot, a Biomek fluorescent plate reader, 3 standing incubators and 2 shaking incubators, Beckman benchtop centrifuge, 4 Fisher brand microcentrifuges, a Statspin cytocentrifuge, 2 CO2 incubators, a Spectramax ELISA plate reader, a Qiagen TissueLyser, vacuum manifold, one -80°C freezer and two -20°C freezers and three +4°C refrigerators.

TOR has eight -80°C freezers and one liquid nitrogen freezer as part of the TOR repository located in the Freezer Faciliity.

RESOURCES

Follow the 398 application instructions in Part I, 4.7 Resources.

Dr. Lieber / University of Washington (UW), Seattle, WA

Laboratory:

FHCRC Biologics Manufacturing Core Lab: The facility occupies approximately 2,600 square feet and is comprised of areas for nonclinical production, clinical production, development, quality control and support space. The Clinical Production Suite (CPS) is the core of the facility and occupies approximately 1,300 square feet divided between two main work areas, production and purification, with related support space for staging, cold room storage, production support and cleanup. Qualified automation systems continuously monitor, control and alarm various aspects within the facility, including: energy management, lab data acquisition, lighting, access (security), fire/smoke alarm, and status of air flow and pressurization of individual spaces within the suite. Keycard access to the facility is restricted to Biologics staff and Center departments providing support services to the facility. The cGMP facility was constructed according to specifications set by Code of Federal Regulations Title 21, Sections 210 and 211. Equipment allows production at scales that exceed the capabilities of most research laboratories and provides the added benefit of clinical cGMP compliance required for non-human primate and eventual clinical studies that would not otherwise be available to investigators. For production of biological reagents, bulk cell capacity can reach up to the equivalent of 100 liters, which is sufficient to produce the amount of JO-1 required for our non-human primate studies. This is carried out in various culture formats, including fully automated fermenter/bioreactor vessels. Equipment is available to harvest either bulk-conditioned media or cells depending upon project needs. In most cases, bulk conditioned media is harvested followed by purification. Purification may consist of multiple steps including affinity, ion exchange, ultrafiltration and diafiltration. In conjunction with these capabilities, the facility maintains a separate well-equipped quality control laboratory to provide characterization and safety testing capabilities

Lieber Laboratory: The laboratory at the UW consists of 750 sq. ft. wet lab. The lab is equipped with all routine molecular biology tools including DNA sequencing equipment, gel and blotting apparatuses, freezers, refrigerators, ELISA reader, flow cytometer, electroporator, centrifuges, etc. Shared equipment includes ultracentrifuges, TaqMan, gamma-counter, immunofluorescence microscope, phospo-imager, triple laser flow cytometer, and *in vivo* imaging system. We currently have a 150 sq. ft. tissue culture room with three hoods, four incubators, inverted microscope, and refrigerator/freezer.

Clinical:

Site for the clinical trial: Swedish Cancer Institute. As a Seattle-area leader in diagnosis, treatment and recovery since 1932, the Swedish Cancer Institute (SCI) cancer care network offers services to help you fight cancer. At the Swedish Cancer Institute, women with ovarian cancer receive care through a nationally recognized gynecological cancer program. This includes the expertise of a renowned team of physicians and other specialists. All ovarian cancer patients at Swedish benefit from a multidisciplinary approach to care that encompasses leading-edge imaging techniques, the latest in treatment options and a full selection of high-quality support services. SCI is also one of the founders of the Marsha Rivkin Center for Ovarian Cancer Research, which is dedicated to saving lives and reducing suffering through improved treatment, early detection and prevention of ovarian cancer.

Animal:

Lieber lab: The mice are housed under SPF conditions in special microisolator units in the vivarium of the K-wing. We have a 500-cage capacity and an adjoining 130 sq ft laboratory equipped with a biosafety cabinet to perform all of the experimental animal procedures described in the grant. All of the surgical equipment and syringes will be housed in the facility. The Washington National Primate Research Center is located in the same building as the PI's lab. This center has both the capacity and the capability to conduct the proposed studies in macaques.

BASi is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) and registered with and inspected by the U.S. Department of Agriculture (USDA-APHIS).

Computer:

There are 8 IBM personal computers and two laser printers in the laboratory.

Office:

The 50 sq ft office with computer is located across the hall from the laboratory.

Other:

Not Applicable

MAJOR EQUIPMENT:

We have access to UW/FHCRC core labs for DNA sequencing, mRNA and miRNA array analyses, confocal microscopy, histology, blood analyses, and in vivo imaging.

RESOURCES

Follow the 398 application instructions in Part I, 4.7 Resources.

Therapeutic Manufacturing Resource

THE FRED HUTCHINSON CANCER RESEARCH CENTER, SEATTLE WA

The Therapeutic Manufacturing resource supports the development and manufacturing of novel biological molecules and innovative cell-based therapies for Phase I/II clinical testing. This resource allows the Consortium to build on it's leadership role in the application of immunotherapy for the treatment of hematological malignancies, and to extend this innovative work to other clinical settings such as autoimmune disorders, melanoma, breast, ovary, and prostate cancer. These facilities permit the reproducible production of large-scale quantities of biological reagents and therapeutic cells under the strict quality control and safety conditions required by the FDA for human studies. Successful development of such materials will have obvious significance for many patients. Further, progress in pursuit of these aims will have relevance for other clinical situations, teaching us valuable lessons about the molecular basis for disease progression, induction of transplant tolerance, response to infectious diseases, and especially about immunotherapy approaches for the treatment of cancer.

Laboratory:
GMP Manufacturing facilities were constructed according to specifications set by Code of Federal Regulations Title 21, Sections 210 and 211. Validation of clean rooms and utilities were conducted in accordance with established standards and facilities have been qualified and utilized for therapeutic manufacturing.
There are two GMP manufacturing sites of 1300 and 4000 square feet respectively. Although each site is primarily dedicated to either biological or cellular products, both facilities have been designed to accommodate both production venues to allow flexibility and maximize product throughput. Non-clinical production, quality control, and other support activities are performed within 2000 square feet of non-GMP laboratory space.

Clinical:
Manufacture and purification of products are performed in dedicated clinical and non-clinical areas of the facility in compliance with regulatory standards. The facility maintains stringent quality control standards for each step of the production process. Production of biological agents includes: monoclonal antibodies, fusion proteins, plasmids, formulation of peptide vaccines, and various other products derived from both eukaryotic and prokaryotic culture systems.

Various analytical methods are utilized to insure that products meet defined release specifications and acceptance criteria. Testing includes; analyses for microbial contaminants (bioburden, sterility, microbial identification, mycoplasma, and endotoxin), biochemical analyses (SDS-PAGE, IEF, ELISA, RID, HPLC, total protein, and osmolality), and/or cell product characterization (cell counts/viability, flow cytometry, phenotyping, purity, colony-forming activity, and functionality). Methods have been validated and elevated to a standard that provides regulatory (FDA) support for clinical studies.

Animal: Not applicable

Computer: Not applicable

Office: Not applicable

Other: Not applicable

MAJOR EQUIPMENT:

Equipment allows production at scales that readily exceed the capabilities of most research laboratories, and as required provides the added benefit of clinical cGMP compliance that would not otherwise be available to

investigators. For production of biologicals, bulk cell culture capacity can range up to the equivalent of 100 liters in various culture formats including fully automated fermentor/bioreactor vessels.

Equipment is available to harvest either bulk-conditioned media or cells depending upon project needs. In most cases bulk conditioned media is harvested followed by purification. Purification may consist of multiple steps including affinity, ion exchange, ultra filtration, and diafiltration.

The resource generally takes a conservative instrumentation approach to allow for maximum product yield in conjunction with moderate capital outlay, ease in instrument repair/maintenance, minimal staffing and simplicity to facilitate Food and Drug Administration (FDA) compliance.

Manufacturing suites supporting the production of cellular therapeutics are equipped with biological safety cabinets and numerous double stack incubators. Other major equipment includes floor and table top centrifuges, cell washers, sterile connecting devices and large scale cell enrichment devices from Baxter and Miltenyi Biotec.

In conjunction with these core equipment capabilities, the Therapeutic Manufacturing Unit maintains a separate well equipped quality control (QC) laboratory space to provide product characterization and safety testing capabilities, such as automated cell counts, flow cytometry, sterility, and endotoxin, as required by the FDA for clinical studies.

All manufacturing activities are conducted using approved written procedures performed by trained, qualified personnel in accordance with cGMP and current Good Tissue Practices (cGTP) guidelines. Quality assurance oversight and review is provided to assure regulatory compliance and safety of therapeutic products.

RESOURCES

Follow the 398 application instructions in Part I, 4.7 Resources.

Hematopathology Laboratory, Department of Laboratory Medicine

THE UNIVERSITY OF WASHINGTON, SEATTLE WA

The primary purpose of the Department of Laboratory Medicine is to serve as a regional resource for clinical laboratory services required for patient care and for educational programs in Laboratory Medicine.

Laboratory Medicine's Hematopathology Laboratory offers a wide variety of testing to aid in the diagnosis of leukemias, lymphomas and other hematopoietic or immunological disorders. In addition, the laboratory is involved in research and development of diagnostic tests and their clinical application. The laboratory personnel also provide high quality teaching to residents, fellows, medical students and medical technology students. Finally, the laboratory is committed to keeping community clinicians informed of new developments in this rapidly changing area of laboratory medicine.

Laboratory:
The Hematopathology Laboratory is a division of the Department of Laboratory Medicine at the University of Washington and occupies ~2500 sq ft of lab space at the Seattle Cancer Care Alliance. The laboratory is a regional reference laboratory and provides diagnostic morphologic, advanced immunophenotyping and molecular services for patient samples derived from both inside and outside the institution. In addition, the laboratory provides technical support for a variety of clinical research activities and is CLIA and CAP certified. The Flow cytometry laboratory is staffed by 16 FTE medical technologists, 8 FTE clerical support staff, and supervised by 8 board certified hematopathologists. The department receives ~20,000 + cases/year.

Clinical: Not applicable

Animal: Not applicable

Computer: Not applicable

Office: Not applicable

Other: Not applicable

MAJOR EQUIPMENT:

The Hematopathology Laboratory has access to all of Laboratory Medicine's equipment including centrifuges, microscopes and flow cytometers.

RESOURCES

Follow the 398 application instructions in Part I, 4.7 Resources.

Swedish Cancer Institute
Swedish Medical Center, Seattle WA

Swedish Hospital Medical Center (SHMC) is the largest, most comprehensive non-profit health care provider in the Pacific Northwest, comprised of three hospital locations in Seattle, one in Edmonds, and one in Issaquah. Swedish has a long-standing tradition of medical excellence. In addition to general medical and surgical care, Swedish is known as a regional referral center, providing treatment of oncology, cardiac care, high-risk obstetrics, orthopedics and organ transplantation (kidney and pancreatic) as well as a number of specialty programs, including eating disorders, sleep disorders, a hospice program and home-care services. The Swedish Cancer Institute (SCI) is the oncology division responsible for all cancer care. It has a primary medical oncology/radiation treatment facility and four satellite radiation clinics. There are also medical oncology clinics at the Ballard, Bellevue, Edmonds, and Issaquah facilities. The SMC saw over 4,095 new analytic cancer cases in 2010.
The SMHC Cancer Institute has long been in the forefront of cancer care in the Pacific Northwest. It is now the largest cancer care program in the Northwest and has over 200,000 square feet of offices and clinical space in five locations. The SCI has nearly doubled its space allowing more room for outpatient clinical services with the latest technologies within the past 10 years.

Laboratory:
Swedish Hospital Medical Center (SHMC) provides services from the CellNetix Pathology and Laboratories, a comprehensive reference laboratory. CellNetix Pathology & Laboratories is a dynamic, rapidly growing private pathology company headquartered in Seattle, Washington, and serving hospitals and clients throughout the greater Puget Sound region. CellNetix provides comprehensive sub-specialty clinical and anatomic pathology services, operates premier anatomic pathology laboratories, and offers complete pathology laboratory services at its central state-of-the-art anatomic pathology laboratory. Cellnetix is known for pathologic excellence based upon pathologist sub-specialization, for valuing intellectual capital at all levels of the company, and for delivering top-quality results. It is the largest clinical laboratory in the State and offers the following special services of: histopathology, cytopathology, cytogenetics, immunohistocytochemistry, immunofluorescent antibodies, viral cultures, automated flow cytometry, DNA testing, cytology, therapeutic and forensic drug testing, and pre-natal testing for high-risk pregnancies. CellNetix is committed to enabling the latest in molecular diagnostic testing for patient care. Molecular diagnostics tests available include: fluorescent *in situ* hybridization (FISH), polymerase chain reaction (PCR) and gene microarray analysis among other technologies. Cellnetix is composed of 43 pathologists and over 200 employess. With their size, economies of scale, resources, and volume of referral material, they have created a centralized, state-of-the-art anatomic pathology laboratory leveraging best-in-class information technologies and equipment with lean process design. CellNetix has attracted highly talented professional, technical, and business leaders who provide each individual clinician and patient with focused, personalized service. CellNetix believes that local pathologists who partner with local clinicians and hospitals results in the best possible patient care. Cellnetix has18 Seattle/First Hill based Pathologists who oversee, review, analyze, and initiate further staining studies. CellNetix boasts a sophisticated array of technology infrastructure and information system applications designed to integrate, automate, and standardize operations. These systems fuse state of the art technology with pathology excellence.

Clinical:
Swedish Hospital Medical Center (SHMC) is a regional referral center for the entire Northwest including Alaska. It has 2000 licensed medical doctors, and employs over 7,000 staff. The Center has 59 beds in two general oncology units in the Seattle facility, twelve at the Edmonds hospital, six at the Ballard facility, and six dedicated beds in the Issaquah facility. Its outpatient oncology services at the Cancer Institute locations see over 5,610 new cancer patients a year, with a volume of approximately 34,800 annual medical oncology visits and 43,000 annual radiation visits. SHMC offers bone marrow, kidney, liver, and pancreas transplants. It has

12 general outpatient clinics and three multidisciplinary specialty centers. The SCI is a comprehensive local and regional referral and treatment center for cancer patients seeking treatment and second opinions. The SCI provides a multidisciplinary clinical setting for treatment of all cancers through the medical oncology, radiation oncology and surgery departments. The SCI facilities include 10,500 square feet dedicated for research offices and activities spread over four campuses. The SCI facilities include physicians' offices, general usage examination suites, special procedures suites, dressing rooms, pharmacies with specialized investigational drug support services, laboratory, five medical treatment centers, radiation treatment, planning areas, radiation therapy and treatment rooms, chest x-ray suites, patient education centers and an integrated care program offering naturopathic care services of art, music, and massage therapy, social services, individual and group counseling, financial counseling, nutritional counseling, ACS Resource Navigator Services, a cancer rehabilitation program, a genetic counseling program, and a meditation program.

Medical Oncology

Seventeen full-time and two part-time medical oncologists maintain clinics across the five medical oncology clinics of the Cancer Institute. Special features include five dedicated Infusion Centers where lengthy chemotherapy treatments can be carried out in a supportive environment. All infusion centers contain rooms with chairs or beds (for long term infusions), telephones, TV's, VCR's and provide a comfortable setting for outpatients and family members. Auxiliary nursing, dedicated Investigational Drug Service pharmacies, nutrition and social work services are available for support to these areas. The Cancer Institute Medical Oncology Clinics contain chest x-ray equipment, in office laboratory facilities, and general pharmacy services to make patient visits easy and efficient. All clinics are supported by a total of 37 registered nurses and 2 nurse practitioners. Ninety percent of the nurses are ONS certified and the Infusion Centers have altogether a total of 32 registered chemotherapy certified nurses.

Radiation Oncology

The SCI was a pioneer in the introduction of high voltage radiotherapy in the United States. In 1932, the first "supervoltage" roentgen therapy apparatus in the United States was installed for radiation therapy in the SCI. In the 1980s and 90s, SCI physicians performed much of the pioneering work in prostate seed implants and high dose rate brachytherapy for prostate cancer. Currently, the field of radiation oncology is in a period of rapid scientific advancement with new technologies providing radiation oncologists with unprecedented capabilities in the areas of tumor imaging, treatment planning, and treatment delivery. The Swedish Cancer Institute (SCI) has been an early adopter of many of these new technologies including intensity modulated radiation therapy (IMRT), image guided radiation therapy (IGRT), volumetric modulated arc therapy (VMAT), Calypso 4D localization system for prostate cancer, partial breast irradiation, CT imaging, robotic radiosurgery, combined PET-CT imaging for radiation treatment planning, lung saving brachytherapy, and real-time intra-fraction tumor tracking. Radiation oncology treatment methodologies include CyberKnife and Gamma Knife radiosurgery capabilities and a tomotherapy unit. The Center for Advanced Targeted Radiation Therapy is a program focused solely on the work of exploring, developing, and testing investigational methods for delivering advanced targeted radiation therapy for cancer patients and disseminating the benefits of these activities to clinicians so cancer patients have early access to new, life-saving treatments. The SCI has continued to invest in the latest technological advances in radiation oncology. Over the past five years, SCI has been among the first cancer centers to adopt a number of new technologies, including:

- Image guided radiation therapy (IGRT): The SCI has invested in three Elekta Synergy treatment units that include cone-beam CT capabilities making it possible to obtain a CT image set of the patient immediately before treatment to ensure accurate patient positioning.
- Electromagnetic localization: The SCI was the world's first commercial installation of the Calypso Medical's 4D localization system which uses wireless transponders implanted in tumors to provide real time feedback on the tumor location throughout treatment delivery.
- 4D CT imaging: The SCI was an early adopter of 4D imaging that allows physicians to visualize the motion of lung tumors over a patient's breathing cycle resulting in improved design of each patient's treatment plan.
- Beam gating: This automatic breathing control system, acquired by Swedish in 2005, can control a patient's breathing during radiation treatment of breast cancer, leading to reduced damage to healthy tissues in the lungs and heart

- PET/CT imaging: The SCI was one of the first radiation oncology departments with a dedicated PET/CT scanner, making it possible to show fused image data sets with both physiological (PET) and anatomical (CT) data.
- Robotic radiosurgery: The SCI acquired a CyberKnife radiosurgery system in 2006. The CyberKnife delivers hypofractionated radiotherapy uses a compact linear accelerator mounted on a computer-controlled robotic arm.
- Electronic Brachytherapy: The SCI was one of the first three institutions in the U.S. to implement the Xoft Axeent, a new technology for delivering partial breast irradiation using a miniaturized x-ray tube.
- CyberKnife and Gamma Knife: Robotic radiosurgery systems to precisely deliver radiation to nearly any part of the body allowing for accurate dose delivery to the target area with minimal exposure to surrounding healthy tissue.

The SCI radiation facilities include five satellite locations. State of the art equipment is used to provide the most up to date treatments and treatment planning available. Included are; a superficial therapy machine, one ortho voltage machine, equipment for stereotactic radiosurgery, 7 linear accelerators, advanced brachytherapy intraarterial equipment, a unit for colorectal irradiation, high-dose rate equipment, CyberKnife and Gamma knife units, mammosite equipment, and 4 treatment simulators. Sixteen radiation oncologists, nine physicists, nine dosimitrists, and thirty three technical staff members make up the radiation oncology division. The clinic saw 1,933 new patients in 2010 with an average daily census of 193.

Surgical Oncology

The SCI developed a Thoracic Surgery Program in 2005 and opened a new Thoracic Surgery clinic in 2008 where four surgeons, two thoracic surgery fellows, a pulmonologist and a nurse practitioner provide a full range of general thoracic surgery services, including benign and malignant conditions. The clinic has six exam rooms, state of the art technology, and easy access to other care modalities. The program includes a lung cancer screening program, the latest in equipment, such as the endobronchial ultrasound, a new minimally invasive procedure for diagnosing lung cancer that has been available at Swedish since early 2007, and the advanced treatment option of VATS, or video-assisted thoracic surgery for early stage lung cancer.
The Breast Cancer Program has five fulltime breast surgeons augmented by 2 part-time surgeons and a nurse practitioner and is the largest breast surgical group in the Seattle area. Their outpatient activities are integrated with the established medical and radiation oncology activities of the SCI Clinics on all five campuses. Their inpatient and operating activities are all undertaken at the same campuses where 1224 new breast cancer cases were seen over the past year.

Hospital Facilities

Inpatient facilities at SHMC include dedicated oncology beds at Edmonds (12), Ballard (10), Issaquah (6) and one 30 bed, and one 29 bed unit at First Hill that include 35 private suites. The First Hill units include five lead-lined rooms for patients with implanted radiation sources. All nurses at all locations are chemotherapy certified. The level of advanced training and communication within these areas assures protocol compliance for the research participants. All stem cell transplantations are completed on the First Hill units. Nurses receive special education prior to treating these patients. Two oncology clinical nurse specialists, two nutritionists, six social workers and a psychiatrist are available to all patients. Surgical services are provided at all facilities in both ambulatory and inpatient operating rooms with full anesthesia care, pre and post operative care and inpatient surgical nursing care.

Animal: Not applicable

Computer: Not applicable

Office:
Ten thousand five hundred (10,500) square feet of office space in the SCI facilities is dedicated to research, nursing and data management. Office furniture and equipment include high-volume photocopiers, dedicated FAX machines, two dedicated scanners, reference materials, filing cabinets, storage, and high- volume shredding capacity. Compaq Pentium II Desk pro computers and HP laser-jet printers are available to this

project. Computers have network access to laboratory, radiology, and pathology reports as well as other pertinent patient information.

Other:

SCI Breast Imaging Center

SCI Breast Imaging Centers network encompasses four screening and comprehensive diagnostic sites, two mobile mammography units with digital mammography and partnerships with primary and specialty care clinics throughout the Puget Sound region. The mobile coaches travel throughout western Washington to screen women in remote areas and underserved urban communities. Annually, the network records over 60,000 screening and diagnostic mammograms and ultrasounds, 2,000 minimally invasive biopsy procedures and 1,000 additional interventional procedures. Stereotactic core biopsies are performed for non-palpable breast lesions. Dedicated, high-density ultrasound machines are available to evaluate mammographic and palpable abnormalities. The diagnostic program offers state-of-the-art mammography equipment. Ultrasound guided fine-needle aspirations and core biopsies are available. Galactography for evaluation of nipple discharge is also offered as well as pre-surgical needle localization of non-palpable lesions. Patient support services include educational programs, resource materials, and breast cancer risk assessment along with genetic testing, psychosocial counseling and second opinion referrals. A computerized patient tracking system follows all patients. The breast program enables access to new breast imaging technologies of PEM (positron emission mammography), 3-D ultrasonography, and breast MRI.

Cancer Patient Support Services

The SCI offers a wide array of services in the Integrated Care Program to complement patients' traditional medical care. Services available are: two American Cancer Society Navigators to assist the patient with any need; art therapy; a hereditary cancer clinic; cancer rehabilitation program; massage therapy; music therapy; meditation; naturopathic care; nutrition care clinic; and the psycho-oncology program staffed with a psychiatrist, and 5 social workers. Nutritional counseling is available to all patients and the social workers provide housing for out-of-town patients, transportation, financial assistance and individual counseling. The Sustaining Care Program provides a smooth transition between the hospital and home care, facilitating comprehensive, supportive services to patients living in the region through an interdisciplinary team of nurses, physical therapists, occupational therapists and social workers. The Home Infusion department provides treatments for patients in home environments. Community partnerships with Gilda's Club, Cancer Lifeline and the American Cancer Society provide classes and emotional support for cancer patients and their families.

First Hill Diagnostic Imaging

This facility collaborates with SCI physicians to provide excellent and state-of-the-art diagnostic services for patients. It has two Siemens MRIs ("magnets":) 1) Advanto(R) 1.5T and 2) Harmony(R) 1.0T. Each has a dedicated breast coil and additionally the Harmony has MRI-guided Biopsy capability. The clinic also has Confirma CADstream(R) and Invivo DynaCAD(R) software and reading stations for computer aided MRI Interpretation and MRI-guided Biopsy targeting. Invivo Biopsy coil and apparatus (either grid or pillar and post) is used as well as either Atec Suros (R) or SenoRx EnCore(R) vacuum-assisted core-biopsy instruments. CADstream is usually used for Interpretation and Image processing while either CADstream (SureLoc(R)) or DynaCAD can be used for MRI-guided targeting for biopsy.

MAJOR EQUIPMENT:

Not Applicable

RESOURCES

Follow the 398 application instructions in Part I, 4.7 Resources.

Pacific Gynecology Specialists

SWEDISH MEDICAL CENTER, SEATTLE WA

Laboratory: Not applicable

Clinical:
The office of Pacific Gynecology Specialists (PGS) is located on the First Hill (FH) campus of SMC. PGS consists of 7 board certified or eligible gynecologic oncologists, 2 board certified gynecologists, and 3 physicians' assistants. In 2012 the gynecologic oncologists performed primary surgery on 180 women with ovarian cancer and managed over 1200 cycles of chemotherapy. The clinic includes a 6 bed infusion suite where patients receive cancer treatments and supportive therapy. The nursing staff are oncology trained with years of experience in chemotherapy administration. Because of their community education approximately 25% of ovarian cancer cases diagnosed by PGS oncologists are in an early stage. These physicians also see approximately 10-12 patients per month for consultation regarding ovarian cancer risk management. PGS associates combined perform over 500 operations for benign ovarian disease, and provide routine gynecological care to over 1,000 women annually. PGS physicians actively support gynecologic research and enroll large numbers of patients into the following: Gynecological Oncology Group (GOG), Puget Sound Oncology Consortium (PSOC) and pharmaceutical company sponsored research trials. Since 1998, they have participated in biological specimen collection protocols which have provided specimens to basic science researchers and have been the major source of biological specimens for the POCRC.

Animal: Not applicable

Computer: Not applicable

Office:
The office of Pacific Gynecology Specialists (PGS) is located on the campus of Swedish Medical Center First Hill (SMC-FH). SMC-FH is the primary cancer treatment hospital in the northwest region providing oncology services for over 110 incident ovarian cancer cases per year.

Other: Not applicable

MAJOR EQUIPMENT:

Not applicable

A. SPECIFIC AIMS

Our goal is to overcome treatment resistance in ovarian cancer patients. A central resistance mechanism is the maintenance of tight junctions between malignant cells that prevent drug penetration into the tumor. We have generated a small recombinant protein (JO-1) that binds to desmoglein 2 (DSG2), a junction protein that is overexpressed in ovarian cancer. Binding of JO-1 to DSG2 triggers signaling pathways that are reminicent of an epithelial-to-mesenchymal transition (EMT) and results in transient opening of tight junctions in epithelial tumors, including ovarian cancer xenografts. We have shown that the intravenous injection of JO-1 increases the intratumoral penetration and efficacy of monoclonal antibodies and chemotherapeutic drugs in a broad range of human xenograft models as well as in human DSG2 transgenic mice with syngeneic epithelial tumors. Further studies have shown that the effective doses of chemotherapy can be reduced when the chemotherapy drugs are combined with JO-1. Finally, our studies have demonstrated that combining JO-1 with chemotherapy drugs markedly reduced the toxic side effects of chemotherapy. The application of JO-1 has not been associated with toxicities in human DSG2-transgenic mice and monkeys, mostly due to the fact that in normal epithelium DSG2 is not readily accessible to intravenously injected JO-1.

The central goal of this proposal is the clinical translation of JO-1 in combination with PEGylated liposomal doxorubicin (PLD) / Doxil® for ovarian cancer therapy. In this context, we have performed pre-clinical efficacy and safety studies in mouse models of ovarian cancer with JO-1 and PLD. We have submitted a pre-IND application (see Appendix #1) which has recently been reviewed by the FDA. Based on the FDA's recommendations, we now have clear guidelines for the next steps in completing the IND package. The FDA's suggestions for JO-1 manufacturing, preclinical toxicity studies, and the clinical trial have been addressed in the research plan of this proposal. Our goal is to submit a full IND application by Year 2.

Specific Aim 1. Evaluate the safety of JO-1 / PLD administration in two preclinical models to support an IND filing:

1.1. cGMP manufacturing of JO-1. A manufacturing protocol for JO-1 at a large scale has already been established. cGMP manufacturing and quality control of a clinical lot of JO-1 will be performed by the FHCRC Biologics Manufacturing Core. The final product will meet cGMP standards and will be used for GLP toxicology studies and the clinical trial.

1.2. GLP toxicology studies with clinical product in human DSG2 transgenic mice and non-human primates. A dose-escalation toxicology study in hDSG2 transgenic mice will be conducted with the clinical JO-1 lot (alone and in combination with PLD) by a GLP certified testing facility. As a second species for toxicology studies, we have selected *Macaca fascicularis.* We demonstrated that DSG2 biodistribution in *Macaca fascicularis* is similar to that in humans and that JO-1 binds to monkey DSG2, triggering opening of junctions in monkey cancer cells. The treatment scheme will be the same as in the clinical trial. Hematological parameters, JO-1/PLD pharmacokinetics, and anti-JO-1/PLD immune responses will be monitored followed by a full necropsy.

Specific Aim 2. Evaluate the safety and efficacy of JO-1 administration in combination with PLD in ovarian cancer patients.

2.1. Evaluate safety. The primary goal of this trial is to confirm the safety of JO-1 when administered in combination with PLD in patients with progressive, persistent or recurrent ovarian/fallopian tube cancer, who have previously received standard therapies. We plan to have 5 patients per cohort with 3 dose levels of JO-1 and a fixed (standard) dose of PLD. We will treat patients for four cycles (treatment given every 4 weeks). The trial will be coordinated through the POCRC Clinical Core and include study sites at the UW/SCCA and the Swedish Cancer Institute at Swedish Medical Center in Seattle. We expect that up to 15 patients will be required.

2.2. Evaluate therapeutic efficacy. Tumor response will be assessed using clinical examinations, serum levels of CA125 and HE4 prior to each cycle of therapy, and imaging (CT or MRI) prior to every other cycle of therapy as well as at the end of the treatment period.

2.3. Gain additional knowledge about the biological effects of JO-1 in patients. We will use the blood samples and analyze *i)* JO-1 pharmacokinetics, *ii)* serum DSG2 concentration, *iii)* anti-JO-1 immune responses (antibodies, T-cells), *iv)* anti-tumor T-cell responses, *v)* metabolic serum markers, and *vi)* circulating tumor cells. If available, ascites/biopsy samples will be included in these analyses.

Clearly, preparing the IND package, conducting the trial and analyzing patient samples requires more resources than the SPORE project can provide. We have therefore obtained letters of support from biotech and biopharma companies, stating that matching funds will be provided (included in Section I below).

B. RESEARCH STRATEGY

B.1. Significance

PLD treatment in ovarian cancer: PLD is a PEGylated, liposome-encapsulated form of doxorubicin. The effective size of the liposome is ~100nm. PLD is used to treat patients with ovarian cancer that has progressed or recurred after platinum-based chemotherapy. Although commonly used to treat recurrent ovarian cancer, response rates to PLD are low, the response duration is short, and the toxicity is significant. The overall response rate in a study with 239 patients was 19.7% (3.8% and 15.9% complete and partial response respectively) (www.doxil.com). Progression-free survival (PFI) was roughly 29 weeks and 9 weeks for patients with platinum sensitive and platinum resistant disease, respectively. A recent Phase 3 trial demonstrated a modest improvement in PFI (11.3 vs 9.3 months, p=.005) and no change in overall survival for PLD-carboplatin *vs*. Paclitaxel/carboplatin combination chemotherapy in patients with recurrent platinum-sensitive ovarian cancer. Side effects of treatment include mucositis, hand-foot syndrome, myelosuppression, cardiotoxicity and liver impairment. When used as mono-therapy for recurrent epithelial ovarian cancer at doses of 40-50mg/m^2, roughly 30% of patients develop a grade 3 toxicity of some type. We decided to test JO-1 (for its first application in humans) in combination with PLD because it has a major role in ovarian cancer therapy and because there is a large body of experience with this drug. Furthermore, new ovarian cancer therapeutics are often tested in combination with PLD (see ClinicalTrials.gov).

Epithelial phenotype of cancer: Greater than 80% of all cancer cases are carcinomas, formed by the malignant transformation of epithelial cells. One of the key features of epithelial tumors is the presence of intercellular junctions, which link cells to one another and act as barriers to the penetration of molecules with a molecular weight of >400 daltons (Da) (13, 21, 23). Several studies have shown that the upregulation of epithelial junction proteins correlated with increased resistance to therapy, including therapy with the two major classes of cancer drugs - monoclonal antibodies and chemotherapeutics (12, 22, 26). One of these junction proteins, desmoglein 2 (DSG2), is upregulated in malignant cells (5, 15). For example, in more than 60 ovarian cancer biopsies with different histological types analyzed, we consistently found higher expression levels of DSG2 in ovarian cancer cells than in the surrounding normal tissue or tumor stroma cells (3). As an example, Figure 1A shows epithelial junctions marked by DSG2 on a biopsy from a patient with serous ovarian cancer. For most carcinomas, progression to malignancy is accompanied by a loss of epithelial differentiation and a shift towards a mesenchymal phenotype, *i.e.* epithelial to mesenchymal transition (EMT) (40). EMT increases migration and invasiveness of many cell types and is often one of the conditions for tumor infiltration and metastasis. However, following invasion or metastasis, cells that have undergone the process of EMT can also revert to a well-differentiated epithelial phenotype (9). In support, there exist numerous examples of advanced carcinomas showing that mesenchymal cells can regain characteristics of epithelial cells or undergo mesenchymal to epithelial transition (MET) (9). As an example, Fig.1B shows DSG2 immunohistochemical analysis of a breast cancer metastasis in the liver.

In summary, the epithelial phenotype of ovarian cancer cells and their ability to form physical barriers protect the tumor cells from attacks by the host-immune system or from elimination by cancer therapeutics (9).

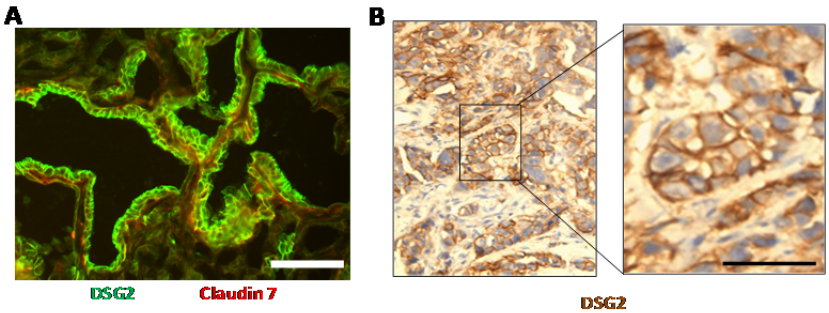

Figure 1. DSG2 is present in epithelial junctions of ovarian and breast cancer. ***A)*** *Immunofluorescence analysis of an ovarian cancer biopsy. The section was stained with antibodies against the junction proteins DSG2 (green) and claudin 7 (red).* ***B)*** *DSG2 immunohistochemistry on a section of a metastatic breast cancer lesion. DSG2 staining appears in brown. The scale bars are 20μm.*

Adenovirus serotype 3 derived junction opener JO-1: We have recently reported that a group of human adenoviruses uses DSG2 as a receptor for infection (46). Among DSG2-targeting viruses is serotype 3 (Ad3). Ad3 is able to efficiently breach the epithelial barrier in the airway tract and infect airway epithelial cells. This is achieved by the binding of Ad3 to DSG2, and subsequent intracellular signaling that results in transient opening of tight junctions between epithelial cells. We have capitalized on this mechanism and created a recombinant protein that contains the minimal structural domains from Ad3 that are required to open the intercellular junctions in epithelial tumors. This protein is called "junction opener 1" or "JO-1". JO-1 is a self-dimerizing recombinant protein derived from the Ad3 fiber (45) (Fig.2A). JO-1 has a molecular weight of

approximately 60 kDa. It can be easily produced in *E. coli* and purified by affinity chromatography. Binding of JO-1 to DSG2 triggers autocatalytic cleavage of DSG2 and activation of pathways that are reminiscent of an epithelial-to-mesenchymal transition (EMT), including the phosphorylation of MAP kinases and the downregulation of junction proteins (4, 44, 46). Both mechanisms result in transient opening of epithelial junctions (Fig.2C).

We have shown in over 25 xenograft models that the intravenous injection of JO-1 increased the efficacy of cancer therapies, including many different monoclonal antibodies and chemotherapy drugs, in a broad range of epithelial tumors (2, 4). Further studies have shown that the effective doses of chemotherapy can be reduced when the chemotherapy drugs are combined with JO-1. Furthermore, our studies have demonstrated that combining JO-1 with chemotherapy drugs markedly reduced the toxic side effects of chemotherapy. The application of JO-1 was safe and well-tolerated in toxicology studies carried out in human DSG2-transgenic mice and a limited number of macaques (4, 46).

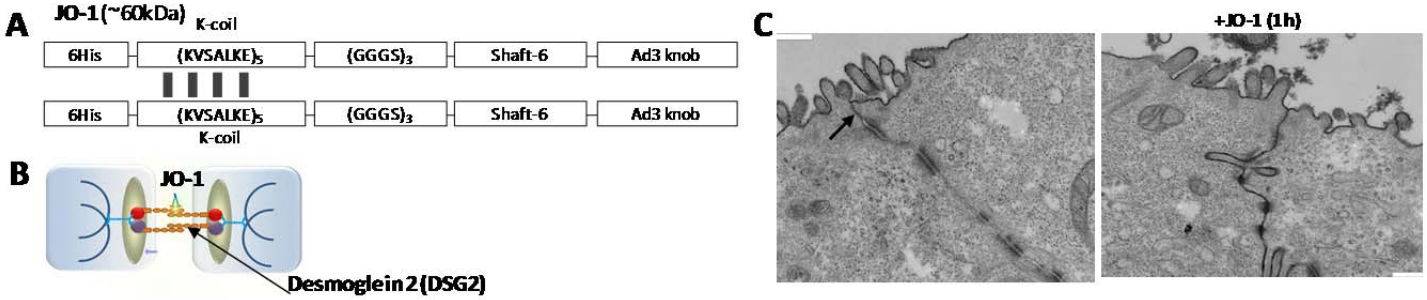

Figure 2. Transient opening of epithelial junctions by JO-1 in vitro. A) *Schematic structure of JO-1 containing an N-terminal His-tag, a dimerization domain [K-coil (48)], a flexible linker, one fiber shaft motif, and the homotrimeric Ad3 fiber knob domain. JO-1 is produced in E-coli (at a yield of ~10 mg/l) and purified with Ni-columns.* ***B)*** *Simplified diagram showing the structure of epithelial junctions with desmosomes and DSG2, the receptor for JO-1.* ***C)*** *Transmission electron microscopy of junctional areas in polarized colon cancer T84 cells. Cells were either treated with PBS (left panel) or JO-1 (right panel) for 1h on ice, washed, and then incubated for 1h at 37 ^{o}C. The electron-dense dye ruthenium red (1) was then added together with the fixative. If tight junctions (above the desmosomes, marked by an arrow) are closed, the dye only stains the apical membrane (black line). If tight junctions are open, the dye penetrates between the cells and stains the baso-lateral membrane. The scale bar is 1μm. Magnification is 40,000x.*

Previous experience in humans: There is no previous human experience with JO-1. JO-1 contains the fiber knob domain of adenovirus serotype 3 and binds to DSG2 in a way similar to Ad3. This implies that studies performed with Ad3 or Ad3 fiber knob containing Ads are relevant for predicting potential side effects of JO-1 in a clinical trial. For more than a decade, Ad5 vectors that possess Ad3 fibers (Ad5/3), which also infect cells through DSG2 (45), have been used for cancer therapy in animal models and humans with a very good safety profile after intravenous injection (20, 31). A recent phase I trial demonstrated safety of an intraperitoneally applied Ad5/3-based oncolytic vector in recurrent ovarian cancer patients (17). Last year, we performed a study with an oncolytic vector based on Ad3 (16). In this study, twenty-five patients with chemotherapy refractory cancer were treated intravenously with a fully serotype 3-based oncolytic adenovirus Ad3-hTERT-E1A.The only grade 3 adverse reactions observed were self-limiting cytopenias. Neutralizing antibodies against Ad3 increased in all patients. Signs of possible efficacy were seen in 11/15 (73%) patients evaluable for tumor markers. Particularly promising results were seen in breast cancer patients and especially those receiving concomitant trastuzumab. The latter might suggest that Ad3 acts in a similar way to JO-1 on tumor junctions and increases drug penetration.

The central hypothesis of the proposal is that the relatively large size of PLD (~100nm) impedes efficient intratumoral penetration in epithelial ovarian tumors and that the combination of JO-1 and PLD can overcome this problem allowing for increased anti-tumor efficacy. Furthermore, based on our preclinical studies as well as the experience with Ad3 fiber containing viruses, we anticipate that intravenous injection of JO-1 is safe in humans.

Significance: PLD is used to treat patients with ovarian cancer that has progressed or recurred after platinum-based chemotherapy. Response rates to PLD are low, the response duration is short, and the toxicity is significant. There is a great need for improvement of both the efficacy and the safety of PLD therapy in ovarian cancer patients.

B.2. Innovation

There are no epithelial junction openers used clinically for cancer therapy. A number of chemical detergents, surfactants, calcium-chelating agents and phospholipids have been used to increase drug absorption through the GI tract epithelium. Recently, Kytogenics Pharmaceuticals, Inc. has developed a tight junction opener based on chitosan derivatives. It is thought to act by electronegative forces applied to tight junction proteins (http://www.kytogenics.com). However, all of these agents act indiscriminately to mechanically disrupt junctions and are therefore unable to be used systemically. Our therapeutic, JO-1, is a small protein that can be produced in *E.coli* and easily purified. It binds to DSG2, which is accessible in epithelial cancer cells and triggers an epithelial-to-mesenchymal-transition that results in transient opening of epithelial junctions.

B.3. Progress Report

In the past we have received the following funding through the SPORE:

- DRP: "***Development of a marker panel for the prediction of oncolytic adenovirus performance in patients with ovarian cancer."*** 2/1/04-9/30/04: We demonstrated that the epithelial phenotype confers resistance of ovarian cancer cells to currently used oncolytic adenoviruses based on serotype 5 and that thus far little-explored adenovirus serotypes Ad3, Ad7, Ad11, and Ad14 can overcome this obstacle by triggering an EMT (8, 36, 37). In the context of this project, we have also established cultures from 65 ovarian cancer biopsies, seven of which can be passaged as xenograft tumors in mice. These culture have been used in studies outlined in section B.4.
- CDP: ***"Identification of markers for specific for cancer stem cells."*** 3/1/06-2/28/07": We have identified subpopulations of ovarian cancer cells from biopsies that are in a transitory epithelial-mesenchymal stage. These cells are not homogenous but contain subsets that can be distinguished based on a number of phenotypic features, including the subcellular localization of E-cadherin, and the expression levels of Tie2, CD133, and CD44. A cellular subset with membrane E-cadherin(low)/cytoplasmic E-cadherin(high)/CD133(high), CD44(high), Tie2(low)) ws highly enriched for tumor-forming cells and displays features which are generally associated with cancer stem (33-35).
- DRP: ***"Identification of genetic alterations that confer resistance to cisplatin therapy in ovarian cancer."*** 8/1/06-7/31/07: We have shown that ovarian cancer cells gain stem cell features through a mesenchymal-to-epithelial transition (MET) and that cisplatin chemotherapy triggers MET in primary human ovarian cells. We have screened a library of small molecules that has recently been generated to influence EMT/MET signaling in induced pluripotent stem cells and found two compounds that blocked MET in ovarian cancer cultures and increased their chemosensitivity.
- CDP: ***"ImmunoGene Therapy of ovarian cancer."*** 4/1/08-3/31/09: We showed that the combination of tumor site-located CTLA-4 blockade and systemic regulatory T-cell depletion induces tumor-destructive immune responses (24, 30, 41, 43). Furthermore, we demonstrated that in situ adenovirus vaccination engages T effector cells against cancer cells.
- DRP: ***"A combination therapy of JO-1 and chemotherapy in ovarian cancer."*** 7/1/11-6/30/12: The outcome of the studies supported by this grant is described in section B.4a-1 or has been published (2, 44).
- Project 3 (year 5): ***"Safety and pharmacokinetics studies after repeated intravenous JO injection (alone and in combination with PLD) in Macaca fascicularis."*** 7/1/13-6/30/14: We have completed a 3-day study with a single JO-1 injection and found no critical side effects (see below: Fig.7 and section B4a-3). A 16-week study with 4 injections of JO-1 +PLD will be started at the Washington National Primate Research Center in October 2013.

B.4. Approach

B.4a. Preliminary data with JO-1 and PLD supporting the clinical trial. The studies that yielded the data shown below have been supported by the NCI (Supplement for R01- CA144057, DRP-FHCR Ovarian Cancer SPORE), the DoD (Translation Pilot Award/Ovarian Cancer Research), and Samyang Biotech (Gift).

B.4a-1. JO-1 enhances PLD chemotherapy in xenograft models of ovarian cancer. To support this proposal, we evaluated PLD in xenograft models with ovarian cancer cells (ovc316). Ovc316 cells are derived from a patient and closely model the heterogeneity and plasticity seen in tumors *in situ* (35). Mice with pre-established ovc316 tumors were intravenously injected with JO-1, followed one hour later by an intravenous injection of PLD (Doxil™). The ability of JO-1 to open up intercellular junctions increased the penetration and amount of PLD in tumors. Immunofluorescence analysis of tumor sections from JO-1/PLD treated animals showed PLD distributed over a greater distance from blood vessels (Fig.3A). More than 10-fold higher amounts

of PLD per gram tumor tissues were measured by ELISA when PLD was combined with JO-1. The enhancing effect of JO-1 on PLD therapy was shown in a model with ovc316-derived mammary fat pad tumors (Fig.3B). Notably, the tumors were resistant to the PLD dose used in this study. The second model was an orthotopic model with intraperitoneal ovc316 tumors (Fig.3C). While all mice treated with PLD alone died by day 80 after tumor inoculation, the combination of JO-1 and PLD resulted in long-term survival (>160 days) of ~80% of animals. Additionally, we have shown JO-1 efficacy in xenograft models of breast, lung, prostate, gastric, colon and skin cancer models (2, 4) (also see pre-IND application/Appendix 1).

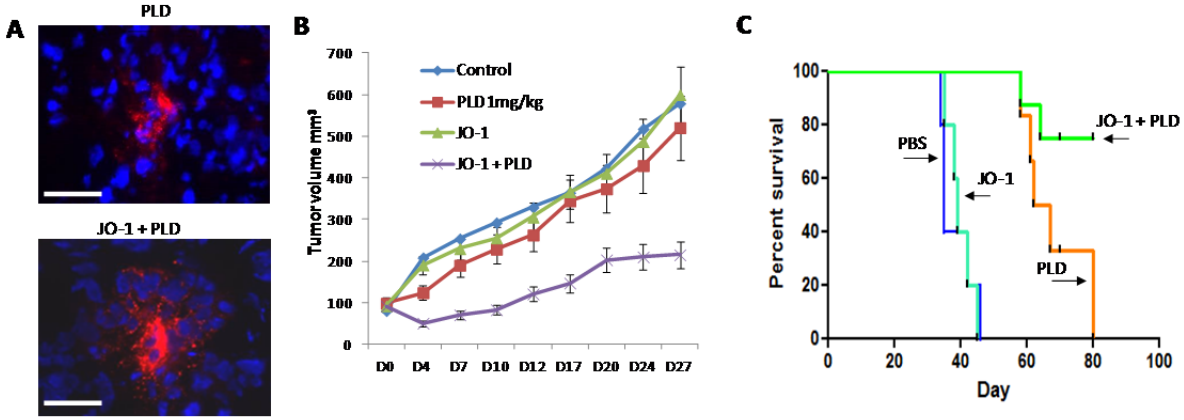

Figure 3. JO-1 enhances PLD therapy in an ovarian cancer model. A) *Better penetration and accumulation of PLD in tumors. Immunfluorescence analysis of tumor sections 2 hours after intravenous PLD or JO-1/PLD injection. PLD is stained red using antibodies that specifically bind to PEG in nanoparticles. The scale bars are 20 μm.* ***B)*** *Efficacy study/mammary fat pad model.Treatment was started when tumors reached a volume of 100 mm^3. Mice were injected intravenously with 2 mg/kg JO-1 or PBS, followed by an intravenous injection of PLD (1 mg/kg) or PBS one hour later. Treatment was repeated weekly.* ***C)*** *Survival study/intraperitoneal model. CB17-SCID/beige mice with intraperitoneal tumors derived from primary human ovarian cancer cells ovc316. Treatment was started at day 25 after tumor cell implantation and repeated weekly. Mice were injected intravenously with 2 mg/kg JO-1 or PBS, followed by an intravenous injection of PLD/Doxil (1 mg/kg) or PBS one hour later. Onset of ascites was taken as the endpoint in therapy studies. Shown is the survival of animals in a Kaplan Meier graph. n=10. p <0.001 for PLD vs JO-1 + PLD.*

B.4a-2. JO-1 enhances PLD chemotherapy in immunocompetent models.

Tumor-specificity of JO-1 action: Because JO-1 does not bind to mouse cells (44), we generated human DSG2 (hDSG2) transgenic mice that expressed human DSG2 at a level and in a pattern seen in humans (44). Furthermore, we established syngeneic tumor cell lines (TC1 and MMC (30)) with ectopic hDSG2 expression at levels seen in human tumors (2). In epithelial tissues of hDSG2 transgenic mice, hDSG2 is specifically localized to junctions (44). Furthermore, JO-1 triggers junction opening in hDSG2-expressing epithelial mouse tumor cells indicating that hDSG2 interacts with mouse signaling and cytoskeletal proteins thus overriding a potential function of mouse DSG2 in maintenance of junctions in transgenic mice. (Notably, there are no mouse DSG2-specific antibodies available.) Using hDSG2 transgenic mice, we demonstrated that JO-1 predominantly accumulates in tumors (2). A number of factors could account for this finding, including: *i)* overexpression of hDSG2 by tumor cells, *ii)* better accessibility of hDSG2 on tumor cells, due to a lack of strict cell polarization compared to hDSG2-expressing normal epithelial cells, and *iii)* a high degree of vascularization and vascular permeabilty in tumors. Because of its preferential binding to and action on epithelial junctions of tumors, JO-1 appears to create a “sink” for therapeutic drugs in tumors, which decreases the levels and exposure of these drugs in normal tissues. A low level of hDSG2-specific JO-1 accumulation was also detected in the small and large intestine, specifically in intestinal epithelial cells that underwent cell division which potentially allowed for access to DSG2 (44).

Effect of JO-1 on biodistribution of gold nanoparticles in hDSG2 transgenic mice. We are currently studying the effect of JO-1 mediated junction opening in epithelial tumors and tissues of hDSG2 transgenic mice by measuring the uptake of PEG-modified gold nanoparticles (AuNP) with diameters of 20 and 120nm (Fig.4A). Notably, the average diameter of PLD is 100nm. We produced Au-NP and confirmed their size by dynamic light scattering. The biodistribution of AuNPs in tissue samples is quantified by inductively coupled plasma mass spectrometry (ICP-MS) at the Environmental Health Laboratory at the University of Washington (Seattle, WA). This method is highly sensitive. So far, we have measured the Au-NP amount in tumors (Figs.4B and C).

Studies on other tissues are ongoing. In tumors, we found that the accumulation of 120nm Au-NP is at least 3 orders of magnitude less efficient than the accumulation of 35nm particles. The amount of 120nm AuNP per gram tumor tissue increased >10-fold when mice were pre-injected with JO-1. We are currently analyzing the AuNP penetration on tumor sections using transmission electron microscopy. We expect that the studies with AuNP will further corroborate that epithelial junctions represent a barrier to intratumoral accumulation of larger drugs (such as PLD) and that this can in part be overcome by JO-1.

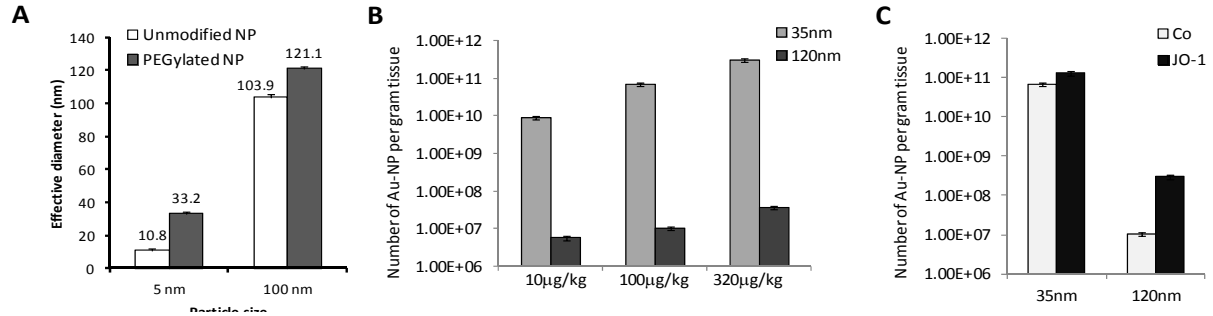

Figure 4. Accumulation of differently sized PEGylated gold-nanoparticles (Au-NP) in tumors. A) *Particle sizing of gold nanoparticles by dynamic light scattering (DLS) before and after modification with $mPEG_{5000}$-SH. Data are presented as mean ± standard deviation, N = 6. 5 nm were from Ted Pella, Redding, CA. 100nm particles were from Nanopartz, Inc., Loveland, CO.* ***B)*** *hDSG2 transgenic mice with syngeneic mammary fat pad MMC-hDSG2 tumors (800-1000mm^3) were intravenously injected with Au-NP at different doses. Six hours later blood was flushed from the circulation and tumors were harvested. Shown are the numbers of Au-NP particles per gram tumor. N=3.* ***C)*** *hDSG2 transgenic mice with established tumors were intravenously injected with PBS (Co) or JO-1 (2mg/kg) followed by an intravenous injection of Au-P at a dose of 100ug/kg. Tumors were harvested 6 hours later. N=3*

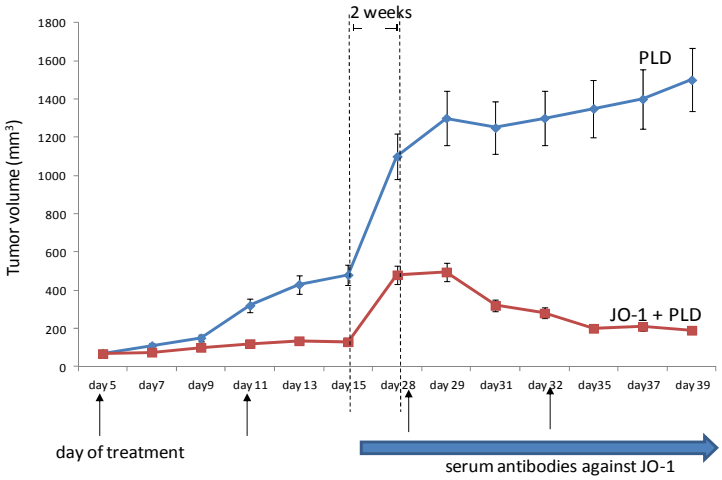

Figure 5. JO-1/PLD therapy in immunocompetent hDSG2 transgenic mice. *A total of $4x10^6$ MMC-hDSG2 cells were injected into the mammary fat pad of hDSG2 transgenic mice. When tumors reached a volume of ~80 mm^3, JO-1 (2 mg/kg) or PBS was injected intravenously followed one hour later by PLD (i.v. 1.5 mg/kg). Treatment was repeated as indicated at day 5 and 11. Tumors were then allowed to regrow for about 2weeks. At this time, serum was analyzed for anti-JO-1 antibodies. Two more treatment cycles were performed at day 28 and day 32. N=5.*

<u>*Repeated JO-1/PLD treatment:*</u> JO-1 is an adenovirus-derived protein and therefore potentially immunogenic. This might not be a critical issue if JO-1 is used in combination with chemotherapy, which suppresses immune responses to foreign proteins. This expectation is supported by studies with oncolytic adenovirus vectors in which immunosuppression allowed for repeated vector application (6, 10, 39). Furthermore, we have demonstrated that JO-1 remains active *in vitro* and *in vivo* even in the presence of anti-JO-1 antibodies generated by JO-1 vaccination of mice (2). This may be due to the fact that JO-1 binds to DSG2 with a very high avidity thus disrupting potential complexes between JO-1 and anti-JO-1 antibodies. (Notably, JO-1 is a dimer of a trimeric fiber knob, which contributes to the picomolar avidity to DSG2 (45).) To test the potential for anti-JO-1 antibody responses to adversely affect the therapeutic effects of JO-1, we performed repeated injections of JO-1 in an immunocompetent hDSG2 mouse tumor model (Fig.5). After two treatment cycles of JO-1 and PLD, treatment was stopped and tumors were allowed to re-grow. The third and fourth treatment cycles were started on days 28 and 32, respectively. At the time of the third cycle, serum anti-JO-1 antibodies were detectable by ELISA in the JO-1/PLD treated group of animals. However, the antibody levels were about ~10-fold lower than in the group which received JO-1 without PLD. In both the third and fourth treatment cycles JO-1 had an enhancing effect on PLD therapy, demonstrating that JO-1 continues to be effective after multiple treatment cycles even in the presence of detectable antibodies.

JO-1 efficacy in an hDSG2 transgenic mouse model with spontaneous tumors. Transplanted tumors might differ phenotypically from tumors that arise spontaneously. We have therefore cross-bred hDSG2 transgenic mice with *neu*-transgenic (*neu*-tg) mice for 7 generations. Neu-transgenic mice overexpress the rat proto-oncogene *Neu* and develop spontaneous mammary tumors between 4 and 8 months of age (18, 25). The onset of tumor development was similar in double hDSG2/neu transgenic mice. The histology of spontaneous tumors in these mice reflects the main features seen in human breast cancer, specifically strong hDSG2 immunoreactivity in junctions between malignant cells (Fig.6A). As seen in the transplantable tumor models, JO-1 increased the efficacy of PLD treatment (Fig.6B).

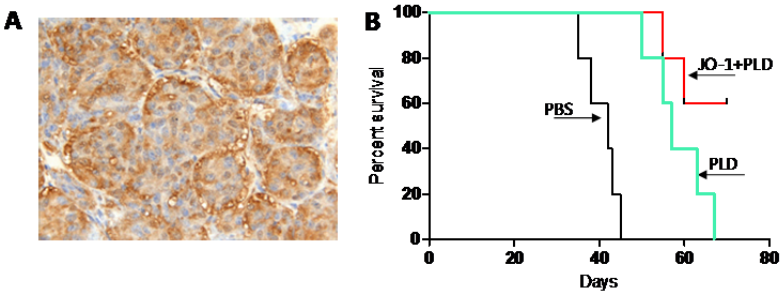

Figure 6. JO-1/PLD therapy in hDSG2/neu transgenic mice with spontaneous tumors. ***A)*** *DSG2 expression in spontaneous mammary tumors.* ***B)*** *Therapy study. As soon as mammary tumors were palpable, JO-1/PLD injection was started. Treatment was repeated weekly. The endpoint in the survival study was >20% loss in body weight due to cancer-related cachexia. N=4*

B.4a-3. JO-1 safety studies. *Safety in hDSG2 transgenic mice:* There were no adverse effects or critical abnormalities found in hematologic and serum chemistry parameters or histopathological studies of tissues after intravenous injection of JO-1 (2 to 10 mg/kg) into hDSG2 transgenic mice (with or without pre-existing anti JO-1 antibodies. We observed a mild lymphocytopenia and intestinal inflammation that subsided by day 3 after injection. We speculate that the favorable safety profile of JO-1 is due to the fact that hDSG2 in tissues other than the tumor and a subset of epithelial cells in the intestine/colon is not accessible to intravenously injected JO-1. The hDSG2 transgenic mouse model was also used to obtain biodistribution and pharmacokinetics data for JO-1. These studies showed a serum half-life of 6 hours and hDSG2-dependent accumulation of JO-1 in epithelial cells of the small intestine and colon (2). We also found hDSG2 dependent binding of JO-1 to peripheral blood lymphocytes and hDSG2-independent uptake by liver, spleen and lymph node macrophages.

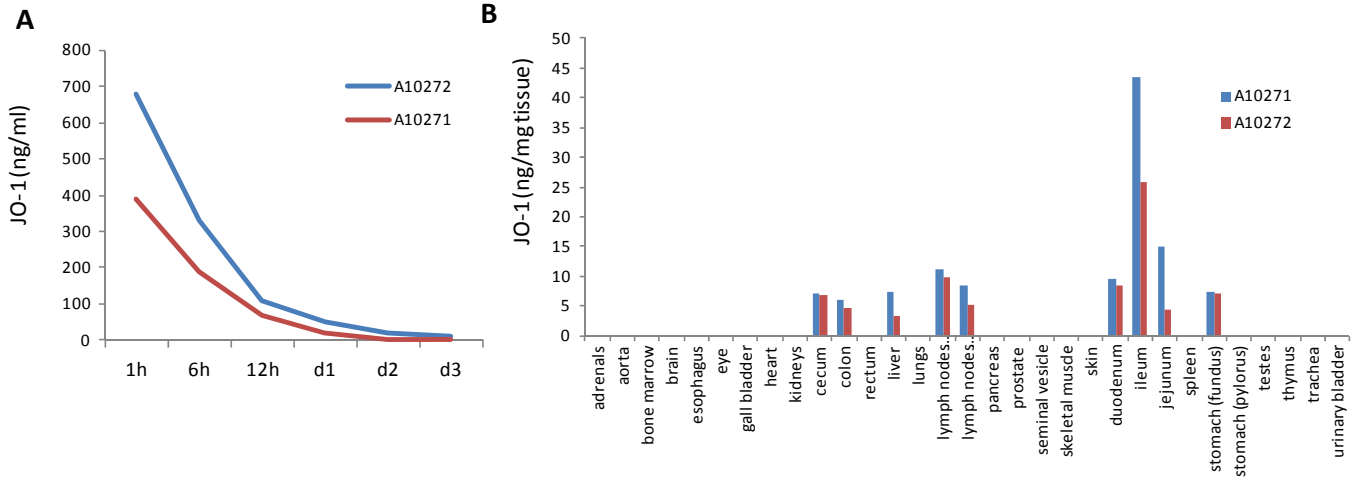

Figure 7. JO-1 levels in macaques afer intravenous injection. A) *Serum levels at different time points after JO-1 infusion. B) tissue levels of JO-1 at day 3 after infusion. A10271 and A10272 were two male Macaca fasciulcaris (~6kg).*

Safety in non-human primates: We demonstrated that DSG2 biodistribution in *Macaca fascicularis* is similar to that in humans and that JO-1 binds to monkey DSG2 (44). So far, two animals have been injected with JO-1 (0.6mg/kg) and monitored for 72 hours, followed by a full necropsy. No laboratory (hematologic or chemistry) or histological abnormalities were observed. JO-1 serum clearance and JO-1 biodistribution was similar to that observed in hDSG2 transgenic mice, i.e . JO-1 was found in the GI-tract as well as macrophages in the liver and lymphnodes (Fig.7). A full pathology report was included in the pre-IND package (Appendix). In September 2013, we will start a small (non-GLP) toxicology study for a combination of JO-1/PLD in *M.fascicularis*. The data can be provided by February 2014.

Risk of inducing metastasis: JO-1 binding to DSG2 on tumor cells triggers pathways involved in EMT, a process which has been associated with tumor metastasis. However, none of our *in vivo* studies have shown

any evidence of increased tumor growth or metastasis after treatment with JO-1. Furthermore, at day 3 after JO-1 injection into mice bearing Her2/neu-positive HCC1954 tumors, there was no significant increase in the percentage of circulating Her2/neu-positive cells in the blood (2). This is likely due to the fact that tumor metastasis requires more than transient activation of EMT pathways. Detachment from epithelial cancers and migration of tumor cells is only possible after long-term crosstalk between malignant cells and the tumor microenvironment, resulting in changes in the tumor stroma and phenotypic reprogramming of epithelial cells into mesenchymal cells (14). In the context of the clinical trial, we will attempt to measure circulating tumor cells before and after JO-1 injection.

Risk of anaphylactic responses: We screened serum samples of 58 ovarian cancer patients (provided by the POCRC) for pre-existing antibodies against JO-1. We found (by ELISA) detectable binding IgG antibodies in less than 10 percent of these samples. No anti-JO-1 IgA, E, and M antibodies were detected. Based on this, one of the inclusion criteria for the clinical trial will be the absence of anti-JO-1 antibodies.

B.4a-4 Preliminary data supporting studies outlined in Specific Aim 2.3.

JO-1 facilitates anti-tumor T-cell responses: Using an immunocompetent hDSG2-transgenic mouse tumor model, we showed that JO-1 injection reduced the number of intratumoral Tregs, which in turn allowed pre-existing anti-tumor CD8 T-cells to control tumor growth (Fig.8). We used hDSG2 transgenic mice and syngeneic TC1 cells that expressed human DSG2. TC1 cells express HPV E6 and E7 and trigger E7-specific T-cells in C57Bl/6 mice. However, these tumor-specific T-cells are unable to control tumor growth and 100% of tumor-bearing animals reached the study endpoint by day 20. Two injections of JO-1 into tumor-bearing mice at days 6 and 14 resulted in complete tumor regression in 60% of mice (Fig.8A). This effect was due to an increased number of E7-specific T-cells (Fig.8B) and could be blocked by systemic depletion of CD8 cells. Importantly, we also found in flow cytometry analyses of tumor-infiltrating leukocytes that the number of Tregs (CD25+/FoxP3+) was about 6-fold less in JO-1 treated animal tumors compared to PBS injected animals (Fig.8C). Notably, JO-1 alone had no significant therapeutic effect in the neu/hDSG2 breast cancer model (Figs. 5 and 6), most likely due to tolerance towards tumor antigens in this model (30).

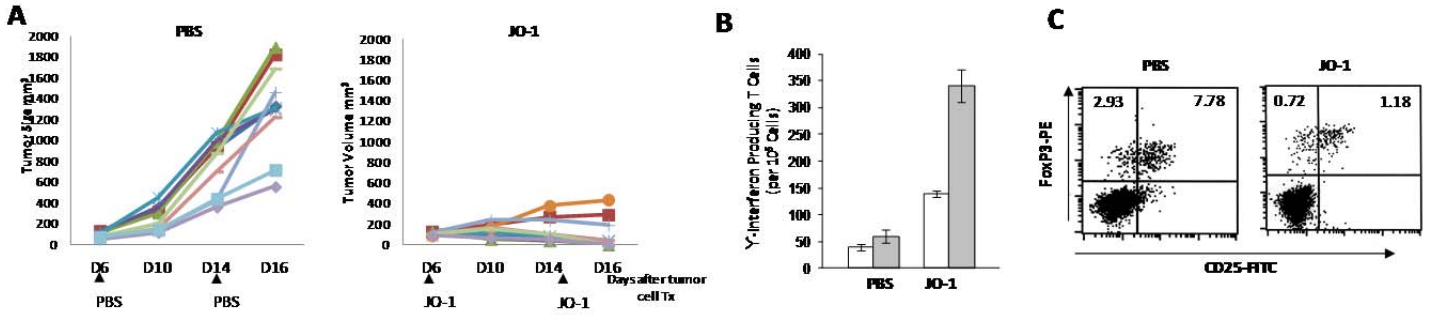

Figure 8. JO-1 facilitates anti-tumor T-cell responses. A) *hDSG2 transgenic mice with established subcutaneous syngeneic TC1-hDSG2 tumors were intravenously injected with PBS or JO-1 (2mg/kg) at days 6 and 14 after tumor cell transplantation and tumor volumes were measured. Each line represents an individual animal. N=10.* ***B)*** *Analysis of frequencies of IFNγ-producing E7 specific T-cells in the spleen 4 days after PBS or JO-1 injection. Shown are the frequencies of IFNγ producing T-cells specific to the HPV16 E749-57 carrying the H-2Db restricted peptide (RAHYNIVTF) (grey bars) or an unrelated control peptide (white bars). N=3 animals per group. The differences in the JO-1 group are significant (p<0.05).* ***C)*** *Analysis of intratumoral (FoxP3+) Tregs 4 days after PBS or JO-1 injection. Shown are representative flow cytometry data.*

We will submit a separate grant application to study the mechanisms behind JO-1 facilitated anti-tumor immune responses. In the context of the clinical trial, we propose to use PBMC to study immune responses.

B.4c Experimental Approach:

Specific Aim 1. Evaluate the safety of JO-1 / PLD administration in two preclinical models to support an IND filing

Hypothesis: Because DSG2 is trapped in junctions of normal epithelial cells, no critical toxicity after intravenous JO-1 injection is expected.

1.1. Manufacturing and characterization of a clinical lot of JO-1. We will produce a single clinical grade cGMP lot of JO-1. The singular lot would be sufficient to first do the GLP toxicology studies while the remaining vialed material is held for subsequent clinical use. JO-1 is produced in E.coli and purified by immobilized metal

chelate affinity chromatography. cGMP production will be performed by the FHCRC Biologics Manufacturing Core (http://sharedresources.fhcrc.org/core-facilities/biologics-production) under the direction of Dr. Ron Manger, who has significant experience in the production of cGMP grade therapeutic proteins. The facility is primarily designed to produce clinical grade proteins intended for intravenous administration in humans. Manufacturing and purification of products are performed in dedicated clinical areas of the facility in compliance with regulatory standards. The facility will also perform the quality control of the produced protein. The protein bulk will be defined and released as bulk Drug Substance. The clinical product will be aseptically filled and released as Drug Product. Drug quality control testing will be done as suggested by the FDA. Details are described in the pre-IND package (Appendix 1). A manufacturing protocol for JO-1 at large scale has already been established. The generation and characterization of a master *E.coli* cell bank has been completed. Furthermore, we demonstrated that after 9 months of storage at -80^{o}C, JO-1 did not lose activity (test ongoing). Therefore, production, quality control, and release of the cGMP lot can be completed within 6 months after the start of funding. The following (already established) *potency assays* of the cGMP product will be performed: *DSG2 binding assay:* The clinical function of the product is to bind to DSG2 triggering intracellular signaling. Binding of JO-1 to DSG2 will be measured by ELISA. Rabbit polyclonal antibodies against DSG2 will be used for capture followed by recombinant DSG2, the cGMP JO-1 lot/reference sample, mouse mAb against the Ad3 fiber knob, and anti-mouse IgG-HRP. *PEG permeability assay*: A total of 5x10^{5} T84 cells will be seeded on 12 mm transwell inserts and cultured for 20 days until the transepithelial electrical resistance (TEER) remains constant, i.e. tight junctions have formed. The cells will be exposed to JO-1 for 15 min at room temperature. 1 μCi of [^{14}C] polyethylene glycol-4000 (MW: 4,000Da) will be added to the inner chamber. Medium aliquots will be harvested from the inner and outer chambers and measured by a scintillation counter. Permeability will be calculated as described elsewhere (47). JO-1 preparations are considered to be functionally potent if they increase PEG permeability >3-fold within 30 min.

1.2. Perform GLP toxicity studies in hDSG2 transgenic mice.

Justification for using hDSG2 transgenic mice for GLP toxicology studies. The homology between the human and mouse DSG2 gene is 77.1% and neither Ad3 nor JO-1 bind to mouse cells (44). We therefore generated transgenic mice that contain the 90kb human DSG2 locus, including all regulatory regions. These mice express human DSG2 in a pattern and at a level similar to humans. Furthermore, we have shown that JO-1 triggers hDSG2-mediated signaling and opening of epithelial junctions in epithelial mouse tumor cells that ectopically express hDSG2 (44) as well as in primary lung epithelial cells from hDSG2 transgenic mice. This indicates that human DSG2 can interact with mouse cytoskeletal proteins and kinases and implies that hDSG2 transgenic mice are an adequate model to study downstream effects of JO-1 binding to DSG2 after intravenous injection.

Design of GLP toxicology study in hDSG2-transgenic mice. While we accumulated a substantial amount of efficacy and safety data in hDSG2 transgenic mice in the PI's lab (see IND package, section 4.5; Figs. 23-31), the FDA requested an additional study at a certified GLP testing facility using JO-1 produced under cGMP compliant conditions. These studies will be performed by Bioanalytical Systems, Inc. (BASi) http://www.basinc.com/about/index.html. Groups #1 to 7 (Table 1) will receive 4 injections of JO-1 and/or PLD 4 weeks apart, based on the clinical protocol. Studies will be performed in naïve animals and hDSG2 transgenic mice that have been vaccinated with JO-1 and have mounted T- and B-cell responses against the protein. Studies in pre-immune animals will address potential allergic side effects of JO-1 injection. The vast majority of preclinical studies performed in the PI's lab were done with a JO-1 dose of 2mg/kg. This will be the starting dose for the GLP toxicology studies. In addition, we will test a "high" dose of JO-1 (4mg/kg). The standard PLD dose in humans is (40mg/m^{2}), which corresponds to a dose of 0.1mg/kg in mice (http://www.fda.gov/cder/guidance/index.htm). For combination treatment, PLD will be injected one hour after JO-1. Groups #8 and 9 will be hDSG2 transgenic mice with hDSG2 expressing tumors derived from the ID8 cells, a spontaneously transformed mouse ovarian surface epithelial cell (29, 38). We have generated ID8 cells expressing human DSG2 at a density that is comparable to that of primary human ovarian cancer cells. These cells form tumors after injection into hDSG2 transgenic mice. Mice will be injected when tumors reach a volume of 100mm^{3} and followed for 4 weeks. Tumor bearing mice reflect better the target patient group in the clinical trial. After each treatment cycle, blood samples will be collected from all animals on day 1 of injection at 3 time points – pre-dose, 6, and 24 hours post-dose, and then twice per week. The samples will be analyzed for: *i)* full blood CBC and chemistry, and *ii)* serum cytokines, *iii)* JO-1 concentrations, *iv)* PLD concentration and *v)* IgG, IgM, IgA, and IgE antibodies against JO-1, PEG and His tag. After 16 weeks, four animals will be euthanized and complete necropsy and histological examination will be performed. Specific attention will be

given to GI-tract and cardiac toxicities. Four animals will be followed post-treatment for 2 weeks with weekly blood analysis. JO-1 and PLD concentrations will be measured in all collected tissues by ELISA. Tissue sections will be analyzed by immunohistochemistry for the presence of JO-1 and PLD. Splenocytes will be used for ELIspot assays to quantitate JO-1-specific γ-interferon-producing CD4 and CD8 T-cells. All assays for safety assessments will be performed under GLPs at the CRO. The exploratory assays for cellular responses are established in the PI's lab.

Animal numbers: For the toxicology studies, we selected a group size of 8 animals. We arrived at this number by setting a large effect size, which was based on our previous studies, and a desired power of 0.8 at a significance level of 0.05.

Table 1: Design of GLP toxicology studies in hDSG2-transgenic mice.

Group	Dosing	Route	Regiment	Duration	N	
1	JO-1 (2 mg/kg) naïve mice, no tumor	IV	Every four weeks	16 weeks	8	4 animals→necropsy 4 animals→follow up
2	JO-1 (2 mg/kg) pre-immune mice, no tumor	IV	Every four weeks	16 weeks	8	4 animals→necropsy 4 animals→follow up
3	JO-1 (4 mg/kg) naïve mice, no tumor	IV	Every four weeks	16 weeks	8	4 animals→necropsy 4 animals→follow up
4	JO-1 (4 mg/kg) pre-immune mice, no tumor	IV	Every four weeks	16 weeks	8	4 animals→necropsy 4 animals→follow up
5	PLD (40 mg/m^2) naïve mice, no tumor	IV	Every four weeks	16 weeks	8	4 animals→necropsy 4 animals→follow up
6	JO-1 (highest tolerated dose) + PLD (40 mg/m^2) naïve mice, no tumor	IV	Every four weeks	16 weeks	8	4 animals→necropsy 4 animals→follow up
7	JO-1 (highest tolerated dose) + PLD (40 mg/m^2) preimmune mice, no tumor	IV	Every four weeks	16 weeks	8	4 animals→necropsy 4 animals→follow up
8	JO-1 (highest tolerated dose) ID8-DSG2 tumors	IV	once	4 weeks	8	4 animals→necropsy 4 animals→follow up
9	JO-1 (highest tolerated dose) + PLD (40 mg/m^2); ID8-DSG2 tumors	IV	once	4 weeks	8	4 animals→necropsy 4 animals→follow up

1.3. Perform GLP toxicity studies in Macaca fascicularis

Justification for using Macaca fascicularis for GLP toxicology studies. JO-1 does not efficiently bind to mouse (44), rat, hamster, and dog PBMC or transformed cell lines (data not shown) and the DSG2 gene homology for these species compared to human DSG2 is less than 80%. Although hDSG2 transgenic mice allow us to study a number of variables in a large number of animals, it is unclear whether the hDSG2-mouse system accurately models a homologous system with human DSG2 in human cells. A better model is non-human primates. The DSG2 gene homology between humans and macaques is 96.6%. Biodistribution in *Macaca fascicularis* is similar to humans (44). JO-1 binds to monkey DSG2 and triggers junction opening at a level that is comparable to human cells (44). This justifies the use of *Macaca fascicularis* for GLP toxicology studies after intravenous JO-1 injection.

Design of GLP toxicology study in Macaca fascicularis. The study will be conducted in accordance with the US FDA Good Laboratory Practice Regulations. The test site will be BASi. The starting dose of JO-1 will be 0.6mg/kg, which corresponds to the dose used in mice (2 mg/kg), after allometric scaling (Table 2). The second cohort will be a "high dose" of 2 mg/kg. The dose of PLD in macaques will be 0.45 mg/kg (corresponds to 40 mg/m^2 in humans). For combination treatment, PLD will be injected 1 hour after JO-1.

Table 2: Design of cGMP toxicology studies in non-human primates.

Group	Dosing	Route	Regiment	Duration	N	
1	JO-1 (0.6 mg/kg)	IV	Every 4	16 weeks	4	2 animals→necropsy 2 animals→follow up
2	JO-1 (2 mg/kg)	IV	Every 4	16 weeks	6	4 animals→necropsy 2 animals→follow up
3	PLD (40 mg/m^2)	IV	Every 4	16 weeks	2	2 animals→necropsy
4	JO-1 (highest tolerated dose) + PLD (40 mg/m^2)	IV	Every 4	16 weeks	6	4 animals→necropsy 2 animals→follow up

Injections will be repeated every 4 weeks. Notably, a recent pilot study in *Macaca fascicularis* did not show serious toxicity of PLD/Doxil following 6 repeated administrations at 2.5 or 4.0 mg/kg (see URL below, "formulation 1" corresponds to Doxil used in patients). Side effects that were observed included scruff, abrasion and skin thickening at injection site, hair loss, moderate increase in RBC, hematocrit, hemoglobin, AST, ALT as well as a moderate decrease in PT and WBCs.

Physical examinations will be performed daily by a qualified veterinarian on all study animals prior to initiation and on all animals prior to their respective necropsy. Body weights will be recorded once prior to treatment and weekly thereafter. Blood samples will be collected and analyzed as described for hDSG2 transgenic mice. After 16 weeks, animals will be euthanized with sodium pentobarbital, exsanguinated, and a gross necropsy will be performed by a certified veterinary pathologist at BASi. A standard set of tissues (~66/animal) will be collected and preserved in 10% formalin. All collected tissues will be examined by a veterinary pathologist. An audited draft of the final report will be available 6 weeks following the final necropsy.
Animal numbers: For non-rodent species, the FDA recommended 4 animals/group for the main study groups, and 2 animals/group for recovery groups.

Specific Aim 2. Evaluate the safety and efficacy of JO-1 administration in combination with Doxil in patients with epithelial ovarian cancer.

In project year 2, we will submit a full IND application for intravenous injection of JO-1 in combination with PLD in ovarian cancer patients. We plan to conduct the trial with Doxil™. We will attempt to secure an adequate supply of the Doxil preparation for our study and consider Lipodox™, another PLD, in the event of shortage of Doxil.

2.1. Documentation of safety: The primary goal of this trial is to confirm the feasibility and safety of JO-1 when administered in combination with Doxil in patients with progressive, persistent, or recurrent ovarian/fallopian tube cancer who have received standard therapies. Three dose levels of JO-1 will be tested in combination with a fixed (standard) dose of Doxil (40mg/m^2). Patients will be treated for four cycles (treatment given once every 4 weeks (standard schedule)) or until toxicity or tumor progression. We chose four cycles of therapy rather than the more typical plan of treatment until disease progression because four treatment cycles *i)* should be sufficient to evaluate for development of toxicity, *ii)* exceed the recommended 3-month duration threshold for repeated dose toxicity trials of anti-cancer treatment required prior to initiation of Phase III trials (ISH S9 Sec 3.4 v4 2009), *iii)* responding patients can continue on Doxil only, and *iv)* treatment to disease progression for all trial participants would be cost prohibitive. *JO-1 dose range:* In the NHP toxicology study we propose to test 0.6 mg/kg and 2 mg/kg. (0.6 mg/kg in macaques = 2 mg/kg in mice after allometric scaling*). The "high" dose in macaques (2 mg/kg) was chosen since we do not anticipate going higher than this dose in any of our human clinical studies. For the clinical trial, the starting and subsequent dose cohorts of JO-1 will be determined based on results from the GLP NHP studies as well as the results of the clinical study. We propose to test three JO-1 doses. Assuming the GLP toxicology demonstrates adequate safety for the 0.6 mg/kg dose in non-human primates we will use that dose as a starting dose. The trial will then be a dose escalation (1.0, 2.0 mg/kg) trial.
Patients will be entered in 3 dose cohorts of 3–6 patients each until the recommended Phase 2 dose (RP2D) is estimated. Intravenous infusion of Doxil will start 1hour after the completion of intravenous infusion of JO-1. The treatment cycle is repeated every 28 days for a total of four cycles or until disease progression or unacceptable toxicity. Patients in the first cohort will be hospitalized and observed for 23 hours after each administration of JO-1/Doxil, and then seen in the outpatient clinic daily during the first 2 days after treatment and then weekly. Patient in the second and third cohort will be observed in MTC (with admission for evidence of any toxicity) and then seen in the outpatient clinic. Standard protocols for monitoring of patients treated with Phase I agents will be followed (Table 3). Blood will be drawn before injection, 1, 2, 3, 4, 6, 12, 24, 48 and 72 hours post-injection (p.i.), and then weekly. Standard safety parameters will be measured including clinical status, physical examination including vital signs, weight, hematologic parameters, serum chemistry studies, coagulation studies and electrocardiograms. Studies in hDSG2 transgenic mice indicated a JO-1 associated decrease in lymphocyte counts. We will therefore pay specific attention to blood cell counts in patients. In addition to potential JO-1-related toxicity, patients will also need to be monitored for toxicities related to Doxil, including infusion reactions, hand-foot syndrome, mucocitis, myelosuppression, cardiotoxicity and hepatic damage. We expect to see less Doxil-associated toxicity, which can be easily evaluated by monitoring the hand-and-food syndrome, in which patients develop painful blistering.

Post-treatment monitoring: In addition to their clinical status, patients will be followed post-treatment for antibodies against JO-1, PEG and His-tag (every 2 months), until antibody levels return to pre-treatment levels. Specific details for the study design, inclusion/exclusion criteria, procedures, clinical and laboratory evaluations, criteria for interrupting the trial, rules for discontinuing injections in an individual subject, rules for suspension of the entire study, and statistical considerations can be found in the pre-IND application, section 2 (Appendix 1).

2.2. Evaluation of therapeutic effects: Although not a primary endpoint of the study, tumor response will be assessed using clinical examinations, serum levels of CA125 and HE4 prior to each cycle of therapy, and imaging (CT or MRI) prior to every other cycle of therapy as well as at the end of the treatment period. For patients with measureable disease, Standard Response Evaluation Criteria In Solid Tumors (RECIST) criteria will be applied to assess a therapeutic response.

Table 3. Monitoring scheme

	Pre-Enrollment	Each Cycle	Day 1#-3 post	Week 1, 2, 3, 4 post
Treatment (JO-1+/-Doxil)		Day 0		
Clinical Analysis				
Clinical status/adverse events	X	X	X	X
Physical examination	X	X	X	X
EKG	X	X	X	
CT or MRI of Abdomen/Pelvis*	X			
Cardiac EF by Echogram or MUGA *	X			
Chest X-ray (CXR)**	X			
Blood collection (CBC,diff, PLTS; Chem 23,	X		X	X
CA125	X	X		
Urine analysis	X	X	X	X
Pregnancy Test	X			
Ascites/Tumor biopsy (if possible)***	X			
Serum antibodies to JO-1****	X	X	X	X
Serum cytokines (5) ****	X	X	X	X
JO-1 in serum ****	X	X	X	X
Anti-JO-1, PEG, 6-His antibodies in serum****	X	X	X	X
Doxil concentrations in serum****	X	X	X	X

Day 1 follow-up evaluation and testing will be performed in hospital prior to discharge.

* Repeated every other cycle. Multi Gated Acquisition Scans will be done before the start of cycle I and after 4 cycles of Doxil to exclude significant cardiac toxicity.

**Repeated every other cycle if initially abnormal

*** Obtain tissue block from a diagnostic surgery or procedure. If patient undergoes biopsy (including para or thorocentesis for clinical indication during treatment we will attempt to collect tissue samples to be used to perform immunohistochemistry for DSG2, and other junction proteins.

**** to be done in PI's lab. Serum cytokine analyses will be performed with BD Cytometric Bead Array (CBA)

2.3. Gain additional knowledge about the biological effects of JO-1 in patients.

***2.3.1 JO-1 and Doxil pharmacokinetics*:** Serum samples will be stored at -80^{o}C. JO-1 concentrations will be measured by ELISA, using rabbit polyclonal anti-Ad3 fiber antibodies for antigen capture and mouse monoclonal anti-Ad3 knob/anti-mouse IgG-HRP for detection (2). The detection limit of this ELISA is 50 pg/ml. Doxil will be measured using an ELISA based on anti-PEG antibodies that we have used earlier (2).

2.3.2. Immune responses:

Binding anti-JO-1 antibodies (IgG, IgM, IgA, IgE) will be measured by ELISA (2). Plates will be coated with rabbit polyclonal anti-Ad3 fiber antibodies, followed by recombinant JO-1, human serum samples (1:2 to 1:1000 dilution), and either anti-human IgG-HRP, anti-human IgM-HRP, anti-human IgG-HRP, or anti-human IgE-HRP. For patients who become seropositive for JO-1, binding antibodies will be monitored until they return

to baseline. *Neutralizing anti-JO1 antibodies (IgG, IgM, IgA, IgE):* The assay will be performed as described previously (4). Human colon epithelial T84 cells will be cultured in transwell plates until transepithelial resistance is constant. JO-1 or PBS mixed with heat-inactivated human serum samples (at a final concentration of 20%) will be added to the inner chamber, followed by ^{14}C-PEG-4,000 1 hour later. Radioactive counts will be measured 1 hour later in the outer chamber. High affinity mouse monoclonal antibodies against Ad3 fiber will be used as a negative control.

Anti-Doxil/PEG antibodies: Doxil consists of doxorubicin HCL encapsulated in STEALTH liposomes coated with an outer layer of methoxypolyethylene glycol (MPEG). An anti-PEG antibody assay will be performed using the commercial kit from ANP Tech:

http://anptinc.com/index.php?option=com_content&view=article&id=124&Itemid=92

JO-1 specific T-cells by ELISpot: PBMCs will be used to evaluate the induction of JO-1- and tumor-specific T-cell responses (7, 19). PBMCs will be isolated by Percoll gradient. Cells will be frozen in CTL-CryoABCTM serum-free media (Cellular Technology Ltd.). For quantification of JO-1-specific T-cells, PBMC will be incubated with JO-1 and control peptides (5 μg/ml) and then subjected to ELIspot assays (h-INF-gammaELIPOST PRO 10 plate kit) as described before (7, 11).

Tumor-specific T-cell immunity by ELISPOT: Preliminary studies in immunocompetent mice indicate that JO-1 increases the frequency of tumor-specific T-cells in the tumor. This observation warrants the analysis of anti-tumor T-cells in the peripheral blood of patients. PBMCs will be used to evaluate the induction of tumor specific T-cell responses (7, 19). ELISPOT will be performed according to MABtech manufacturer instructions (h-INF-gammaELIPOST PRO 10 plate kit). For the anti-tumor response *i)* survivin (BIRC5 PONAB) peptide, *ii)* a pool of CEA+Ny-ESO-1 peptides, *iii)* a pool of c-Myc+SSX2, MAGE-3 and WT-1 peptides (ProImmune), as well as IGFBP peptides (27) will used. Of note, no pre-stimulation of PBMCs will be done in order to avoid artificial or incorrect signals and also to ensure adequate viability of cells which might be compromised during prolonged culture. In addition to these T-cell studies, we will analyze circulating Tregs (FoxP3+, CD4/CD25+) by flow cytometry of PBMCs as described earlier (41, 43). All immunological assays are established in the PIs lab (11, 41, 42).

Expected outcome: Our data shown in Fig.5 indicates that multiple cycles of JO-1/ PLD treatment are effective in immunocompetent animals despite anti-JO-1 serum antibodies. Should we find that anti-JO-1 immune responses affect the safety or efficacy of the approach, we will focus on creating JO-1 variants in which immunodominant epitopes were mutated. Should the studies indicate that JO-1 improves anti-tumor immune response, we will attract additional funding for more detailed mechanistic studies in animal models.

2.3.3. JO-1's action on the tumor

DSG2 shedding: JO-1 triggers autocatytic cleavage of DSG2 in tumors (4), thus increasing the levels of serum hDSG2 in hDSG2 transgenic mice with syngeneic tumors (44). We will therefore measure DSG2 serum concentrations in patient samples by ELISA as described earlier (44). We will also perform immunoprecipitation of serum protein with anti-DSG2 mAbs and analyze the material by Western blot and MS-MS to better understand the mechanisms for JO-1-triggered DSG2 shedding.

Circulating tumor cells (CTCs): Although our studies with xenograft tumor models did not indicate that JO-1 treatment increases CTCs (2), we will address this potent risk factor by measuring CTCs in peripheral blood using the CellSearch system and reagents (Veridex) (32). In this assay, a CTC is defined as EpCAM+, cytokeratin+, CD45-, and is positive for the nuclear stain DAPI. The normal reference range for CellSearch is <2 CTC/7.5 mL of blood. Furthermore, because it is believed that ovarian cancer CTC have undergone an EMT and lost EpCAM expression, we use a CTC detection system based on a broader antibody cocktail that covers epithelial (EpCAM, HER2, MUC1, EGFR) as well as mesenchymal markers (cMET, N-cadherin, CD318) (28).

Studies on biopsy and ascites samples: If tumor biopsies are available, we will perform immunohistochemistry analyses for DSG2 and apoptosis markers, as well as staining for infiltrating immune cells (CD4, CD8, CD68, FoxP3). Selected findings will be validated by Western blot of tumor lysates or by flow cytometry of tumor cell suspensions obtained by collagenase/dispase digest (35). If pre- and post-treatment ascites samples are available, we will include them in T-cell and CTC assays.

Expected outcome: The additional data will help to better understand the JO-1 action of JO-1 on the tumor and will, potentially, create the basis for new treatment approaches that involve JO-1.

C. PROGRESS REPORT PUBLICATION LIST *(*An asterisk denotes each publication that is a result of formal collaborations among different projects within the SPORE, with other SPOREs, or with other funded NCI networks*)

Beyer, I., H. Cao, J. Persson, H. Song, M. Richter, Q. Feng, R. Yumul, R. van Rensburg, Z. Li, R. Berenson, D. Carter, S. Roffler, C. Drescher, and A. Lieber. 2012. Coadministration of epithelial junction opener JO-1 improves the efficacy and safety of chemotherapeutic drugs. Clin Cancer Res 18:3340-3351. PMCID: PMC3547677

Choi, I. K., R. Strauss, M. Richter, C. O. Yun, and A. Lieber. 2013. Strategies to increase drug penetration in solid tumors. Front Oncol 3:193. PMCID: PMC3724174

Liu, Y., S. Tuve, J. Persson, I. Beyer, R. Yumul, Z. Y. Li, K. Tragoolpua, K. E. Hellstrom, S. Roffler, and A. Lieber. 2011. Adenovirus-mediated intratumoral expression of immunostimulatory proteins in combination with systemic Treg inactivation induces tumor-destructive immune responses in mouse models. Cancer Gene Ther 18:407-418. PMCID: PMC3096725

Persson, J., I. Beyer, R. Yumul, Z. Li, H. P. Kiem, S. Roffler, and A. Lieber. 2011. Immuno-therapy with anti-CTLA4 antibodies in tolerized and non-tolerized mouse tumor models. PLoS One 6:e22303. PMCID: PMC3136517

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Strauss, R., P. Sova, Y. Liu, Z. Y. Li, S. Tuve, D. Pritchard, P. Brinkkoetter, T. Moller, O. Wildner, S. Pesonen, A. Hemminki, N. Urban, C. Drescher, and A. Lieber. 2009. Epithelial phenotype confers resistance of ovarian cancer cells to oncolytic adenoviruses. Cancer Res 69:5115-5125. PMCID2738419

Tuve, S., B. M. Chen, Y. Liu, T. L. Cheng, P. Toure, P. S. Sow, Q. Feng, N. Kiviat, R. Strauss, S. Ni, Z. Y. Li, S. R. Roffler, and A. Lieber. 2007. Combination of tumor site-located CTL-associated antigen-4 blockade and systemic regulatory T-cell depletion induces tumor-destructive immune responses. Cancer Res 67:5929-5939.

Tuve, S., Y. Liu, K. Tragoolpua, J. D. Jacobs, R. C. Yumul, Z. Y. Li, R. Strauss, K. E. Hellstrom, M. L. Disis, S. Roffler, and A. Lieber. 2009. In situ adenovirus vaccination engages T effector cells against cancer. Vaccine 27:4225-4239. PMCID: PMC2727281

Wang, H., I. Beyer, J. Persson, H. Song, Z. Li, M. Richter, H. Cao, R. van Rensburg, X. Yao, K. Hudkins, R. Yumul, X. B. Zhang, M. Yu, P. Fender, A. Hemminki, and A. Lieber. 2012. A new human DSG2-transgenic mouse model for studying the tropism and pathology of human adenoviruses. J Virol 86:6286-6302. PMCID: PMC3372198

Wang H, Yumul R, Cao H, Ran L, Fan X, Richter M, Epstein F, Gralow J, Zubieta C, Fender P, Lieber A. Structural and functional studies on the interaction of adenovirus fiber knob domains and desmoglein 2. J Virol. 2013 Aug 14. [Epub ahead of print].

Other program related publications

Wang, H., Li, ZY., Liu, Y., Persson, J., Beyer, I., Möller, T., Koyuncu, D., Drescher, M.R., Strauss, R., Zhang, XB., Wahl, JK, Urban, N., Drescher, C., Hemminki, A., Fender, P., Lieber, A. (2011) Desmoglein 2 is a receptor for adenovirus serotypes 3, 7, 11, and 14. *Nature Medicine*, 17(1):96-104. PMCID: PMC3074512

Wang H, Li Z, Yumul R, Lara S, Hemminki A, Fender P, Lieber A. (2011) Multimerization of adenovirus serotype 3 fiber knob domains is required for efficient binding of virus to desmoglein 2 and subsequent opening of epithelial junctions. *Journal of Virology,* 85(13):6390-402. PMCID: PMC3112237

Beyer I, van Rensburg R, Strauss R, Li Z, Wang H, Persson J, Yumul R, Feng Q, Song H, Bartek J, Fender P, Lieber A. (2011) Epithelial Junction Opener JO-1 Improves Monoclonal Antibody Therapy of Cancer. *Cancer Research,* 71(22):7080-90. PMCID: PMC3217128

Hemminki O, Diaconu I, Cerullo V, Pesonen SK, Kanerva A, Joensuu T, Kairemo K, Laasonen L, Partanen K, Kangasniemi L, Lieber A, Pesonen S, Hemminki A. (2012) Ad3-hTERT-E1A, a fully serotype 3 oncolytic adenovirus, in patients with chemotherapy refractory cancer. *Molecular Therapy,* 20(9):1821-30. PMCID: PMC3437574

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E. HUMAN SUBJECTS

E.1 PROTECTION OF HUMAN SUBJECTS

This Human Subjects Research meets the definitions of a clinical trial.

E.1.1. Risks to Human Subjects

E.1.1.a. Human Subjects Involvement, Characteristics, and Design. This proposal will conduct a Phase I clinical trial of JO-1 when administered in combination with PLD in patients with progressive, persistent or recurrent ovarian/fallopian tube cancer who have previously received standard therapies. The primary objectives of this trial are to: (1) evaluate safety of the JO-1 when administered with PLD, (2) evaluate therapeutic efficacy of the treatment, and (3) gain additional knowledge about the biological effects of JO-1 in patients.

Patients 18 years of age or older with epithelial ovarian, fallopian tube, or primary peritoneal cancer that is persistent or recurrent following primary treatment that includes a platinum and taxane compound with up to two additional therapies are eligible to participate. Patients who have received only one prior cytotoxic platinum based regimen must have a platinum-free interval of less than 6 months, or have progressed during platinum-based therapy, or have persistent disease following a platinum-based therapy. Patients cannot have received prior therapy with PLD or an anthracycline. Patients with detectable pre-treatment anti-JO1 biding antibodies will be excluded.

This trial will enroll 15 subjects unless there is excessive toxicity. Three dose levels of JO-1 will be tested in combination with a fixed (standard) dose of Doxil (40mg/m^2). Enrolled eligible patients will be treated for four cycles on a standard schedule of treatment given once every 4 weeks, or until toxicity or tumor progression. Patients will be entered in 3 dose cohorts of 3-6 patients each until the recommended Phase 2 dose (RP2D) is estimated. Intravenous infusion of Doxil will start 1 hour after the completion of intravenous infusion of JO-1.

Subjects will also have approximately 50 cc blood drawn at various time points: prior to injection, 1, 2, 3, 4, 6, 12, 24, 48 and 72 hours post-injection, and then weekly. Blood drawn will be analyzed for changes in serum chemistries as an assessment of potential toxicity.

Tumor response will be assessed using clinical examinations, serum levels of CA125 and HE4 prior to each cycle of therapy, and imaging (CT or MRI) prior to every other cycle of therapy as well as at the end of the treatment period.

Project activities involving human subjects will take place at the following POCRC collaborating sites:

Swedish Medical Center (FWA00000544)

Marsha Rivkin Center for Ovarian Cancer Research (FWA00005258)

Stanford University Medical Center (FWA00000935)

E.1.1.b. Sources of Material. This research materials obtained from participating subjects include, but are not limited to: (1) medical records to assess eligibility, (2) blood specimens used for clinical evaluation, and (3) research bloods to evaluate KO-1 and PLD kinetics, immunology and biologic effects.

During the course of the study we will record vital signs, results from clinical laboratory and physical evaluations, results of laboratory assays and adverse events self-reported or observed during the study and during follow-up. We will collect basic demographics including race and ethnicity.

Accessing the link between personal identifying information and the research samples are restrictive. The principal investigators are responsible for designating which research staff can access the link. Electronic data will be maintained in a password-protected computer database. This data is backed-up on a regular basis and stored off-site. All hard copy records will be stored in locked file cabinets at all times unless being reviewed.

In addition to the secured files and database, only the coded unique study number and DOB will be provided to those processing the blood samples.

In order to evaluate the results of this study, the following people and government agencies may get access to subject identities: (1) Doctors and research staff taking part in the study, (2) FDA, (3) NIH, (4) Fred Hutchinson Cancer Research Center – Cancer Consortium IRB and (5) POCRC Clinical and Path Cores.

E.1.1.c. Potential Risks. The potential risks may include: (1) Doxil toxicities (2) adverse reactions such as anaphylaxis, (3) venous access for blood tests and treatment infusion, (3) minimally invasive clinical procedures including EKG, CT imaging, Cardiac EF and Chest X-ray, (4) infusion of JO-1 and PLD. The likelihood of these risks are discussed together with procedures for minimizing risk in section E.1.2.b. below.

The alternatives to this study include standard chemotherapy or other approved and experimental therapies available at many medical centers specifically for patients with progressive or recurrent ovarian cancer. These may include: high dose chemotherapy, novel chemotheraputic agents, and immune based treatments.

E.1.2. Adequacy of Protection Against Risks

E.1.2.a. Recruitment and Informed Consent. Subjects will be self-referred or referred by their primary oncologist. This study will be posted on the Fred Hutchinson Cancer Research Center Recruitment Website, the Swedish Cancer Institute Clinical Trials website, and ClinicalTrials.gov. The SPORE Clinical Core will assist in patient recruitment.

Once a patient has been evaluated for eligibility, has been deemed eligible and agrees to pursue participation, they will be sent or given for initial review: (1) the study consent form, and (2) medical records release (HIPAA) form. An appointment will be made for them to meet with a Study Physician, Research Nurse or qualified Research Assistant for an informed consent appointment.

The consenting procedure usually takes 1 to 2 hours with time spent reviewing procedures, the specifics of the research study, risks, benefits and alternative and the specifics of our research group. It is emphasized that this is a phase I study and may be of no clinical benefit to the patient. There is no coercion to enroll and the subjects are assured that a decision to not enroll in no way influences their relationship with their primary oncologist or their ongoing medical care. After all questions are answered and if agreeable, the subjects will sign the consent documents and be provided a copy for their records.

E.1.2.b. Protections Against Risk.

Pregnancy

This study may be harmful to an unborn child. The risks to an unborn child are unknown. Pregnant women are not eligible to participate and a pregnancy test will be performed at the start of the trial, prior to beginning treatment. Women of child bearing potential must agree to use an adequate form of birth control during the entire study period.

JO-1 infusion

Subjects may experience infusion reactions. There is no previous human experience with JO-1 however based on our animal studies we do not expect serious adverse reactions. Except for a mild, transient diarrhea, intravenous injection of JO-1 had no critical side effects on other tissues or hemotologic parameters in hDSG2 mice. We will therefore pay specific attention to blood cell counts in patients. Non-human primate studies showed no laboratory or histological abnormalities. JO-1 binding to DSG2 on tumor cells triggers pathways involved in EMT, a process which has been associated with tumor metastasis, however none of our *in vivo* studies have shown evidence of increased tumor growth or metastasis after injection with JO-1. We will monitor for potential signs of metastasis by measuring circulating tumor cells before and after JO-1 injection. Patient vital signs will be closely monitored in the first 23 hours after injection and they will also be followed post-treatment for antibodies against JO-1, PEG and His-tag every 2 months until antibody levels return to pre-treatment levels.

PLD infusion

Subjects may experience the following common side effects: hand-foot syndrome, mucocitis, myelosuppression, alopecia and nausea. More serious effects may include cardiotoxicity and infusion reactions consisting of shortness of breath, chest pain or anaphylactoid reaction. Doxil toxicity will be

evaluated post-injection by careful monitoring of patients using physicial examination and diagnostic tests such as lab values. Toxicities will be graded according to NCI CTC version 4 and promptly reported. Management of toxicities consists of following the protocol for dose reductions or holding treatment.

Venous Access

Venous access and treatment administration will be provided by a trained professional to minimize the risk of drug extravasation. Although rare, if an extravasation occurs approved hospital procedures will be followed. During blood draws, subjects may experience pain, bruising, lightheadedness, fatigue, or on rare occasions infection. Subjects are encouraged to hydrate prior to blood draws. If they experience any side effects related to an intravenous treatment or blood draw site, they are instructed to contact one of the participating research study staff to describe their symptoms/reactions.

EKG

Subjects may develop a mild rash where the electrodes (soft patches) were attached. This rash often disappears without treatment.

CT of Abdomen/Pelvis

Risks of CT scans are allergies to the contrast dye and exposure to radiation, however the risk from one scan is small. The most common contrast given in a vein contains iodine. Patients who are allergic to iodine may experience nausea or vomiting, sneezing, itching or hives. Patients will be asked about iodine allergies prior to the scan and if an allergy is suspected will be given antihistimines prior to the test. Risk of anaphylaxis is rare. Subjects will be closely monitored during the procedure and if there are any serious problems the procedure will be stopped.

Chest X-ray

Chest x rays have few risks and the amount of radiation is small. A lead apron will be used to protect certain parts of the patient's body from radiation.

Confidentiality

The link between the subject and their personal health information and the unique study ID number are restricted to research staff designated by the Principal Investigator and Co-Investigator who will ensure confidentiality and managed according to the requirements of the Health Insurance Portability and Accountability Act of 1996 (HIPAA).

E.1.3. Potential Benefits of the Proposed Research to Human Subjects and Others.

Risks associated with this study are reasonable as there are limited options for patients with progressive or recurrent ovarian cancer following primary treatment. The benefits to this study are unknown. The treatment may help the subject's condition or it may not. It will, however, benefit patients with cancer in the future as we will gain substantial knowledge from this first in humans trial.

E.1.4. Importance of Knowledge to be Gained.

PLD is currently used to treat patients with ovarian cancer that has progressed or recurred after platinum-based chemotherapy, however response rates are low, response duration is short, and the toxicity is significant. A central resistance mechanism is the maintenance of tight junctions between malignant cells that prevent drug penetration to the tumor. Adding JO-1 to PLD treatment may cause transient opening of these tight junctions, allowing more of the drug to reach the tumor and potentially reducing the amount of drug needed for effective therapy thereby reducing toxic side effects.

E.1.5. Data Safety and Monitoring Plan

The Co-Principal Investigators (PIs) are responsible for every aspect of the design, conduct and final analysis of the protocol. Regulations defining the responsibilities for assessment and report of all adverse events (AE), serious AE and unexpected AE are defined by the Code of Federal Regulations: 21 CFR 312.32 and Common Terminology Criteria for Adverse Events (CTCAE) Version 3.0 published by the Cancer Therapy Evaluation Program (CTEP), a division of the NCI/NIH.

This clinical study will rely upon the monitoring of the trial by the Principal investigators in conjunction with a Co-investigator, Research Nurse, Research Assistant, Statistician, an independent Medical Monitor and an

Independent Study Monitor assigned by the Clinical Trials Support Office of the Fred Hutchinson Cancer Research Center. .A Medical Monitor will meet with the P.I.s prior to subjects being recruited in order to review the conduct and monitoring mechanisms for the study. The Medical Monitor will then meet bi-annually throughout the data collection period with the P.I., Study Physician(s), P.A.-C, Research Nurse(s), Research Assistant(s) and other applicable clinical research staff members as determined by the P.I.s. The Medical Monitor will review subject recruitment and retention, adherence to protocol, follow-up, data quality, and participant risk versus benefit. If a serious adverse event occurs the P.I.s will be notified immediately and Medical Monitor will be notified along with the IRB and appropriate federal agencies (i.e. FDA).

Adverse events will be monitored as: Patients are monitored for the development of end organ damage by assessing adverse events with EKG, serum chemistries, liver function studies, complete blood counts and physical exams performed every visit with evaluation beginning after the first treatment. All adverse events for all systems are graded on a scale of 1-5 and attribution is assigned, using the Common Terminology Criteria for Adverse Events. If an adverse event occurs during more than one cycle of treatment, then only the most severe adverse event is reported. All information pertaining to toxicity is recorded on the source document.

Procedure for reporting adverse events:

a. Identify the classification/attribution of the adverse event as defined above using the NCI Common Terminology Criteria for Adverse Events.
b. After appropriate medical intervention has been instituted, the Principal Investigator or his/her designee will be notified within 24 hours.
c. File appropriate reports immediately by phone/fax with appropriate agencies, as described above.
d. Notify the patient's primary physician or referring physician within a medically appropriate timeframe, depending on the classification of the adverse event.
e. Submit written reports to appropriate agencies.
f. Document the adverse event in the patient's study chart, using a progress note to describe the event and treatment, if appropriate.
g. Copies of all forms/correspondence relating to the adverse event would be filed in the patient's study chart.

Agency Reporting Requirements:

Fred Hutchinson Cancer Research Center: Cancer Consortium IRB – Expedited adverse event reporting should be reported to the IRB as soon as possible but within 10 calendar days.

Expedited adverse events must meet all three reporting requirements:

1. Unexpected
2. Related or possibly related to the research procedures
3. Serious, meaning it resulted in any of the following outcomes: death, life-threatening event, inpatient hospitalization or prolongation of existing hospitalization, persistent or significant disability/incapacity/or change in psychosocial status, a congenital anomaly, or requires intervention to prevent permanent impairment or damage.

Attach the most current consent document(s).

The PI or designee must sign the Expedited Reporting Form for Unanticipated Problems or Noncompliance.

Submit the Expedited Reporting Form for Unanticipated Problems or Noncompliance and Adverse Event Reporting Form directly to the Institutional Review Office (J6-110). Submit only the original documents. If documents are being modified, submit a Research Modification Form and modified documents, if applicable.

FDA (for trials using an Investigational New Drug (IND)) – As the sponsor of the IND for this study we will follow 21 CFR Part 312 for safety reports to the FDA. In summary written reports are submitted to the FDA for an event that is Related to the product and Serious and Unexpected using the FDA Form 3500A MedWatch form. The safety report may be called or faxed to the appropriate contact for this IND as soon as possible but in no event later than 7 calendar days.

NCI - As the Sponsor of the IND for this study we will follow the controlling regulations of the Food and Drug Administration as required by the NCI. Thus, all adverse events observed during the study will be reported to

the NCI in the form of a progress report. Temporary or permanent suspension of an NCI funded clinical trial is to be reported to the NCI grant program director responsible for the grant.

E.1.6. ClinicalTrials.gov. If this project is funded we will register the trial with ClinicalTrials.gov.

E.2. INCLUSION OF WOMEN AND MINORITIES

All participants in this ovarian cancer research study will be women. Members of any race will be included if they present with eligibility based on the criteria listed elsewhere. The FHCRC and affiliated institutes continue to be challenged to meet accrual goals for under-represented groups.

In general, the population pool from which we will be drawing our study group is 94.4% Caucasian, 2.3% Asian American, 1.8% American Indian/Alaskan Native, and 1% African American. The Targeted/Planned Enrollment Table shows the gender and ethnic breakdown.

Targeted/Planned Enrollment Table

This report format should NOT be used for data collection from study participants.

Study Title: Combination Therapy of JO-1 and Pegylated Liposomal Doxorubicin in Patients with Epithelial Ovarian Cancer

Total Planned Enrollment: 15

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	**Females**	**Males**	**Total**
Hispanic or Latino	1	0	1
Not Hispanic or Latino	14	0	14
Ethnic Category: Total of All Subjects *	15	0	15
Racial Categories			
American Indian/Alaska Native	0	0	0
Asian	2	0	2
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	1	0	1
White	12	0	12
Racial Categories: Total of All Subjects *	15	0	15

* The “Ethnic Category: Total of All Subjects” must be equal to the “Racial Categories: Total of All Subjects.”

E.4. INCLUSION OF CHILDREN

Children aged 18-21 who meet eligibility requirements are eligible for this trial.

E.5. Key Personnel Training in the Use of Human Subjects in Research

The leaders of the project and all key personnel involved with human subjects research within this project have completed approved institutional training in the Use of Human Subjects in Research that covers the following content:

1. Introduction and Historical Perspective
2. Ethical Principles
3. Regulations and Process
4. Informed Consent
5. Research with Investigational Biologics, Drugs and Devices
6. Behavioral Research
7. Research with Protected Populations: Prisoners
8. Research with Protected Populations: Minors
9. Research with Protected Populations: Pregnant Women and Fetuses in Utero
10 Research with Protected Populations: Vulnerable Subjects: A Definition
11. Records-Based Research
12. Genetic Research
13. Research Integrity

F. VERTEBRATE ANIMALS

Mice at the University of Washington

All animal procedures at the UW will be performed under the supervision of the University of Washington Institutional Animal Care and Use Committee. The studies are covered by our IACUC protocol #3108-1 (PI: Lieber). Animals will be housed in the UW Animal Facility, located in the K-wing (-2 level) of the Magnuson Health Sciences Building.

We will provide to BASi:

-35 naïve hDSG2 transgenic mice (6-12 weeks old)

-35 preimmune hDSG2 transgenic mice

-20 hDSG2 transgenic mice with hDSG-ID8 tumors (mammary fat pad, 100mm^3)

1. Experimental procedures

Human DSG2 transgenic mice: Because the mouse orthologue of DSG2 is not recognized by JO-1, toxicology studies in normal mice are relatively inadequate. We have therefore generated C57Bl/6-based transgenic mice expressing human DSG2 in a pattern and at a level similar to those found in humans. Furthermore, we showed that JO-1 binding to human DSG2 in transgenic mouse epithelial cells triggered junction opening. In preliminary studies with DSG2-transgenic mice we did not find significant side effects of intravenous JO-1 injection (2 mg/kg). To create an ovarian cancer model, we will use C57Bl/6-derived ID8 cells (29). ID8 cells are spontaneously transformed mouse ovarian surface epithelial cells that morphologically and immunologically resemble ovarian carcinomas seen in women. We have generated ID8 cells expressing human DSG2 (ID8-DSG2) at a density that is comparable to that of primary ovarian cancer cells.

Procedures:

a) hDSG2-ID8 tumors: A total of 1x10^6 hDSG2-ID8 in 50-100ul of Matrigel (1:1) will be injected into the mammary fat pad under Avertin anesthesia. Tumors will be measured daily. When tumors reach 100mm3, animals will be shipped to BASi. Based on our previous experience, tumors will reach the maximum size 1,000mm^3 in ~8 weeks.

b) intravenous injection of JO-1 to establish pre-immunity. A total of 2mg/kg of JO-1 in 200ul of PBS will be injected into the tail vein.

Table 1: Design of GLP toxicology studies in hDSG2-transgenic mice (based on FDA recommendations).

Group	Dosing	Route	Regiment	Duration	N	
1	JO-1 (2 mg/kg) naïve mice, no tumor	IV	Every four weeks	16 weeks	8	4 animals→necropsy 4 animals→follow up
2	JO-1 (2 mg/kg)	IV	Every four weeks	16 weeks	8	4 animals→necropsy

	pre-immune mice, no tumor					4 animals→follow up
3	JO-1 (4 mg/kg) naïve mice, no tumor	IV	Every four weeks	16 weeks	8	4 animals→necropsy 4 animals→follow up
4	JO-1 (4 mg/kg) pre-immune mice, no tumor	IV	Every four weeks	16 weeks	8	4 animals→necropsy 4 animals→follow up
5	PLD (40 mg/m^2) naïve mice, no tumor	IV	Every four weeks	16 weeks	8	4 animals→necropsy 4 animals→follow up
6	JO-1 (highest tolerated dose) + PLD (40 mg/m^2) naïve mice, no tumor	IV	Every four weeks	16 weeks	8	4 animals→necropsy 4 animals→follow up
7	JO-1 (highest tolerated dose) + PLD (40 mg/m^2) preimmune mice, no tumor	IV	Every four weeks	16 weeks	8	4 animals→necropsy 4 animals→follow up
8	JO-1 (highest tolerated dose) ID8-DSG2 tumors	IV	once	4 weeks	8	4 animals→necropsy 4 animals→follow up
9	JO-1 (highest tolerated dose) + PLD (40 mg/m^2) ID8-DSG2 tumors	IV	once	4 weeks	8	4 animals→necropsy 4 animals→follow up

2. Justify the use of animals

New approaches for cancer treatment with PLD can only be studied *in vivo*. Tumor cell cultures *in vitro* do not reflect the complex interaction between tumor cells and the host. Mice are an adequate model for tumor xenografts to demonstrate bridged activity between two compounds as used in this application.

3. Provide information on the veterinary care of the animals involved.

Animals will be monitored for behavior every day and weighed twice a week. The university will weigh mice daily if there is a question about their health or weight loss. If they encounter weight loss of 20% or other signs of morbidity (absence of grooming, eating or drinking, watery eyes, wet fur, inflammation of sutures), the affected animals will be euthanized. Routine veterinary care is provided by the Department of Comparative Medicine, which oversees the operation of the animal facility. The facilities for animal maintenance and procedure rooms are certified and periodically inspected by the Institutional Animal Care and Use Committee.

4. Describe the procedures for ensuring that discomfort, distress, pain and injury will be limited to that which is unavoidable in the conduct of scientifically sound research.

There are no experimental contradictions to withhold analgesics from animals at any time, although the procedures will be limited to injection of the therapeutic and blood draws. Animals are routinely monitored by the center and if they show signs of distress will be treated or euthanized as deemed appropriate by the attending veterinarian.

5. Describe any method of euthanasia to be used and the reasons for its selection.

Mice will be sacrificed through an Avertin overdose. This method is consistent with recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

6. Animal number selection using statistical power calculations

For the toxicology studies, we selected a group size of 8 animals. We arrived at this number by setting a large effect size, which was based on our previous studies, and a desired power of 0.8 at a significance level of 0.05.

Mice at BASi

BASi is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) and registered with and inspected by the U.S. Department of Agriculture (USDA-APHIS).

JO-1 injection, blood sampling and necropsy will be performed under BASi IACUC protocol #XXX

Procedures:

a) intravenous injection of JO-1 to establish pre-immunity. A total of 2mg/kg of JO-1 in 200ul of PBS will be injected into the tail vein.

b) blood sampling: retroorbital, saphenous vein.

Experimental groups are described in Table 1.

Groups #1 to 7 (Table 1) will receive 4 injections of JO-1 and/or PLD 4 weeks apart, based on the clinical protocol. Studies will be performed in naïve animals and hDSG2 transgenic mice that have been vaccinated with JO-1 and have mounted T- and B-cell responses against the protein. Studies in pre-immune animals will address potential allergic side effects of JO-1 injection. The vast majority of preclinical studies performed in the PI's lab were done with a JO-1 dose of 2mg/kg. This will be the starting dose for the GLP toxicology studies. In addition, we will test a "high" dose of JO-1 (4mg/kg). The standard PLD dose in humans is (40mg/m^2), which corresponds to a dose of 0.1mg/kg in mice (http://www.fda.gov/cder/guidance/index.htm). For combination treatment, PLD will be injected one hour after JO-1. Groups #8 and 9 will be hDSG2 transgenic mice with hDSG2 expressing tumors derived from the ID8 cells, a spontaneously transformed mouse ovarian surface epithelial cell (29, 38). We have generated ID8 cells expressing human DSG2 at a density that is comparable to that of primary human ovarian cancer cells. These cells form tumors after injection into hDSG2 transgenic mice. Mice will be injected when tumors reach a volume of 100mm3 and followed for 4 weeks. Tumor bearing mice reflect better the target patient group in the clinical trial.

After each treatment cycle, blood samples will be collected from all animals on day 1 of injection at 3 time points – pre-dose, 6, and 24 hours post-dose, and then twice per week. The samples will be analyzed for: *i)* full blood CBC and chemistry, and *ii)* serum cytokines, *iii)* JO-1 concentrations, *iv)* PLD concentration, and *v)* IgG, IgM, IgA, and IgE antibodies against JO-1, PEG, and His tag. After 16 weeks, four animals will be euthanized and complete necropsy and histological examination will be performed. Specific attention will be given to GI-tract and cardiac toxicities. Four animals will be followed post-treatment for 2 weeks with weekly blood analysis. JO-1 and PLD concentrations will be measured in all collected tissues by ELISA. Tissue sections will be analyzed by immunohistochemistry for the presence of JO-1 and PLD. Splenocytes will be used for ELIspot assays to quantitate JO-1-specific γ-interferon-producing CD4 and CD8 T-cells. All assays for safety assessments will be performed under GLPs at the CRO. The exploratory assays for cellular responses are established in the PI's lab.

Macaques at BASi

1. Experimental procedures

The study will be conducted in accordance with the US FDA Good Laboratory Practice Regulations. The test site will be BASi. The starting dose of JO-1 will be 0.6mg/kg, which corresponds to the dose used in mice (2mg/kg), after allometric scaling (Table 3). The second cohort will be a "high dose" of 2mg/kg. The dose of PLD in macaques will be 0.45mg/kg (corresponds to 40mg/m^2 in humans). For combination treatment, PLD will be injected one hour after JO-1. Injections will be repeated every four weeks.

Notably, a recent pilot study in *Macaca fascicularis* did not show serious toxicity of PLD/Doxil following 6 repeated administrations at 2.5 or 4.0 mg/kg (see URL below, "formulation 1" corresponds to Doxil used in patients). Side effects that were observed included scruff, abrasion and skin thickening at injection site, hair loss, moderate increase in RBC, hematocrit, hemoglobin, AST, ALT as well as a moderate decrease in PT and WBCs. http://www.ncbi.nlm.nih.gov/pubmed?term=PEGylated%20liposomal%20doxorubicin%20macaques

Table 3: Design of cGMP toxicology studies in non-human primates.

Group	Dosing	Route	Regiment	Duration	N	
1	JO-1 (0.6 mg/kg)	IV	Every four weeks	16 weeks	4	2 animals→necropsy 2 animals→follow up
2	JO-1 (2 mg/kg)	IV	Every four weeks	16 weeks	6	4 animals→necropsy 2 animals→follow up
3	PLD (40 mg/m^2)	IV	Every four weeks	16 weeks	2	2 animals→necropsy
4	JO-1 (highest tolerated dose) + PLD (40 mg/m^2)	IV	Every four weeks	16 weeks	6	4 animals→necropsy 2 animals→follow up

Physical examinations will be performed daily by a qualified veterinarian on all study animals prior to initiation and on all animals prior to their respective necropsy. Body weights will be recorded once prior to treatment and weekly thereafter. Blood samples will be collected and analyzed as described for hDSG2 transgenic mice. After 16 weeks, animals will be euthanized with sodium pentobarbital, exsanguinated, and a gross necropsy will be performed by a certified veterinary pathologist at BASi. A standard set of tissues (~ 66/animal) will be collected and preserved in 10% formalin. All collected tissues will be examined by a veterinary pathologist. An audited draft of the final report will be available 6 weeks following the final necropsy.

Table 2: DSG2 gene homology and binding of the parental virus Ad3 to PBMC from different species. *Ad3 is labeled with ^{3}H thymidine and bound particles can be measured with a scintillation counter. Binding to human PBMCs was taken at 100%.*

Species	DSG2 gene homology	Rel. binding of ^{3}H-Ad3-GFP on PBMC
Human	100%	100%
Chimpanzee	99.0%	N/A
Macaque	96.6%	98%
Mouse	77.1%	5%
DSG2-tg mouse	100%	96%
Rat	76.5%	6%
Hamster	N/A	11%
Dog	78.7%	19%
Cow	76.6%	N/A

2. Justify the use of animals

Justification for using Macaca fascicularis for GLP toxicology studies. JO-1 does not efficiently bind to mouse (44), rat, hamster, and dog PBMC (Table 2) or transformed cell lines (data not shown) and the DSG2 gene homology for these species compared to human DSG2 is less than 80%. Although hDSG2 transgenic mice allow us to study a number of variables in a large number of animals, it is unclear whether the hDSG2-mouse system accurately models a homologous system with human DSG2 in human cells. A better model is non-human primates. The DSG2 gene homology between humans and macaques is 96.6%. Biodistribution in *Macaca fascicularis* is similar to humans (44). JO-1 binds to monkey DSG2 and triggers junction opening at a level that is comparable to human cells (44). This justifies the use of *Macaca fascicularis* for GLP toxicology studies after intravenous JO-1 injection.

3. Provide information on the veterinary care of the animals involved.

A full-time attending veterinarian is on site and maintains the facilities in accordance with AAALAC accreditation standards. In addition, a part-time consultant veterinarian is available when needed. The attending veterinarian is also available for after-hours calls. Animals are observed at least two times each day by facility personnel. Anytime an animal is observed in a condition indicating severe pain or distress, or that may indicate an animal is in a life-threatening condition, it is to be brought to the attention of the attending veterinarian and study director immediately, and appropriate action is taken and recorded in the study record. The proposed studies do not involve surgery.

4. Describe the procedures for ensuring that discomfort, distress, pain and injury will be limited to that which is unavoidable in the conduct of scientifically sound research.

There are no experimental contradictions to withhold analgesics from animals at any time. In the event that any aspect of this study causes more than normally expected brief pain or distress to the animals, the Study Director shall determine, in consultation with the Attending Veterinarian, if administration of appropriate sedatives, analgesics, or anesthetics, or other therapies would be contraindicated by the objectives of the study and document the resultant course of action. Animals that experience severe or chronic pain or distress that cannot be relieved will be euthanized.

The BASi Evansville IACUC reviews the care and use of animals in proposed and ongoing protocols/procedures with respect to compliance with USDA regulations as governed by the Animal Welfare Act (9CFR) and all applicable amendments, AAALAC guidelines as provided in the Guide for the Care and Use of Laboratory Animals (NCR, 1996), and Public Health Service Policy on Humane Care and Use of Laboratory Animals. Protocols/procedures raising concerns from IACUC members and/or study personnel will be reviewed with the study director to discuss the necessity of the protocol/procedure and/or possible alternatives. If the IACUC determines that a protocol/procedure is unacceptable, i.e. causing unnecessary pain and distress, the IACUC will act to prevent the initiation/continuation of the project.

The proposed studies do anticipate the use of restraining devices for intravenous dosing and blood collection purposes.

5. Describe any method of euthanasia to be used and the reasons for its selection.
In the proposed studies, the monkeys will be euthanized by intravenous sodium pentobarbital followed by exsanguination in accordance with the AVMA Guidelines on Euthanasia (June 2007).

6. Animal number selection
For non-rodent species, the FDA recommended 4 animals/group for the main study groups, and 2 animals/group for recovery groups.

G. SELECT AGENT RESEARCH
Not Applicable.

H. CONSORTIUM/CONTRACTUAL ARRANGEMENTS
See attached LOI from the University of Washington.

I. LETTERS OF SUPPORT
See attached Letters of Support.

samyang

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August 2nd, 2013

André M. Lieber, M.D., Ph.D.
Professor of Medicine, Adjunct Professor of Pathology
University of Washington
K-240B Health Sciences Building
Medical Genetics Box 357720
Seattle, WA 98195-7720
USA

Dear Professor Lieber

We are excited to continue our work with you on your JO-1/OPUS therapeutic project as an adjunct therapy for cancer. Samyang has been pleased to provide you with over $45,000 over the past year to produce a sufficient amount of OPUS for pre-clinical tests. Initial studies performed by us indicated that OPUS can increase the therapy of Samyang's chemotherapy drug platform.

Samyang pledges to continue to support your important ovarian cancer research. We will conduct additional tests with various formulations of OPUS to further evaluate its efficacy in improving our chemotherapeutic drug platform under the Supply and Option Agreement of May 15th. Upon receipt of results acceptable to us, we will provide $400,000 in support of your FHCRC Ovarian Cancer SPORE grant project, "Combination therapy of JO-1 and PEGylated liposomal doxorubicin in patients with ovarian cancer".

We wish you continued success in your research endeavors and look forward to productive future collaborations with you.

Yours sincerely

Chulwoong Sohn
Vice President
Samyang Biopharmaceuticals Corporation

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André Lieber
Division of Medical Genetics
Box 357720
University of Washington
Seattle, WA 98195
Phone: (206) 221-3973

July 23, 2013

Dear Dr. Lieber,

This letter is in support of your Ovarian Cancer SPORE proposal "Combination therapy of JO-1 and PEGylated liposomal doxorubicin in patients with ovarian cancer". We are excited that this research proposal is moving forward and want to add our enthusiastic support. We will provide support of up to $400,000 to help translate this project from the preclinical stage to human trials. Our support will be in the form of FTE allocations for process development, regulatory assistance, biochemical and biological assay development and product characterization as well as in cash transferred to defray the cost of outsourced projects.

Protein Advances Inc. is a corporation started in 2004 with the aim of moving benchtop concepts towards commercialization. We have the lab facilities and equipment to do process and assay development that would complement the expertise in the rest of your team.

Our support is there to demonstrate that we as a commercial affiliate have an interest in human validation of the technology and are committed to moving the project on through concept testing and commercialization with help of our corporate partners.

Let me know if there is anything else we can help with.

Best Regards,

Darrick Carter, Ph.D.
Chief Executive Officer
Protein Advances Inc.

UNIVERSITY OF WASHINGTON SCHOOL OF MEDICINE

Department of
Comparative Medicine

T-142 Health Sciences Center
Box 357190
Seattle, WA 98195-7190
Phone: 206-543-8047
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July 25, 2013

Andre Lieber, M.D., Ph.D.
Professor
Division of Medical Genetics
University of Washington
Seattle, WA 98195

Dear Andre,

I have read your Ovarian Cancer SPORE proposal entitled "A combination therapy of JO-1 and PEGylated liposomal doxorubicin in ovarian cancer patients". I would be pleased to collaborate with you on this proposal. In particular I will be able to assist you with the planning and performance of studies focused on selecting a safe dose of your therapeutics.

I have extensive experience in the planning and implementation of pre-clinical studies to support human clinical trials so I'm familiar with developing relevant biomarkers of injury/toxicity. In this regard I have contributed to several IND applications involving cancer therapeutics, including gene-based therapeutics, and I am familiar with the spectrum of pre-clinical studies and the process necessary to support clinical trials. Please let me know when you need assistance.

Sincerely,

Denny Liggitt, DVM, PhD, DACVP
Professor and Chair
Department of Comparative Medicine
School of Medicine
University of Washington

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Gail Pairitz Jarvik, MD, PhD
The Arno G. Motulsky Endowed Chair in Medicine
Professor and Head, Division of Medical Genetics

July 31, 2013

Andre Lieber, MD, PhD
Professor
Division of Medical Genetics, Adjunct Pathology
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Seattle, WA 98195

Dear Andre,

I am delighted to serve as a consultant on your Ovarian Cancer SPORE proposal *"Combination therapy of JO-1 and PEGylated liposomal doxorubicin in patients with ovarian cancer"*.

You can contact me in questions related to the statistical analysis of preclinical and clinical data.

I wish you the best of luck with your application.

Sincerely,

Gail P. Jarvik, M.D., Ph.D.
The Arno G. Motulsky Chair Endowed in Medicine
Professor and Head, Division of Medical Genetics

FRED HUTCHINSON
CANCER RESEARCH CENTER
A LIFE OF SCIENCE

June 18, 2013

Dr Andre Lieber
University of Washington
1705 NE Pacific St.
Seattle WA 98195

Dear Andre,

It would be a pleasure to offer the services of our cGMP manufacturing facility for the manufacture of the JO-1 recombinant protein as described in your ovarian cancer SPORE grant, Project 3: "Combination therapy of JO-1 and PEGylated liposomal doxorubicin in patients with ovarian cancer." I have provided a quote of $82,000.00 that encompasses major aspects of cell banking, assessment of productivity, development of regulatory compliant fermentation and purification processes, final formulation and characterization, and production of cGMP material suitable for intravenous use in NHP and initial clinical studies. Based upon our collaboration to date, we estimate that clinical material we be available within FY14.

I currently manage the Biologics Production Facility at the Center, and our group has considerable experience with the development of both pre-clinical and clinical biological reagents. The facility also provides support with QC/QA testing necessary to produce reagents suitable for *in vivo* use and to comply with regulatory requirements for therapeutic agents intended for clinical use.

Our service also encompasses submission of an associated Drug Master File for your product to the FDA. For my part I would be happy to contribute to the development of this reagent and in the development of methods that would allow compliance with cGMP manufacture of a clinical-grade therapeutic.

Regards,

Ronald Manger

Ronald Manger, Ph.D.
Staff Scientist & Director.
Biologics Production Facility

BASi
Your Scientific Connection

BASI EVANSVILLE
10424 MIDDLE MT. VERNON RD
MT. VERNON, IN 47620 USA

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JDEVINE@BASINC.COM

24 July 2013

André Lieber
Division of Medical Genetics
Box 357720
University of Washington
Seattle, WA 98195
Phone: (206) 221-3973
lieber00@u.washington.edu

Dear André:

I have provided an informal cost estimate for the repeat dose studies in mice and monkeys. As time permits, please review the proposal and return any questions or comments. Once I receive your feedback, I can prepare formal cost estimates.

GENERAL TOXICOLOGY STUDIES

Description of Preclinical Toxicology Fees	Quantity	Subtotal
GLP 16-Week IV Toxicity Study in DSG2 transgenic mice	1	170,500
GLP 16-Week IV Toxicity Study in Cynomolgus Monkeys	1	335,750

GLP 16-Week IV Toxicity Study in DSG2 transgenic mice

- This study will be designed to conform to the FDA Good Laboratory Practice for Nonclinical Studies regulations (CFR 21 Part 58).
- DSG2 transgenic mice will have DSG2 expressing tumors derived from the ID8 cells, a spontaneously transformed mouse ovarian surface epithelial cell (25, 35) – supplied by sponsor
- 10/sex/group main study animals (80 main study animals). An additional 5/sex/group will be assigned to the control and high-dose for recovery (20 recovery animals) and 9/sex/group will be assigned to Groups 2-4 for TK collections.
- Physical examinations, including ophthalmology, will be performed by a qualified veterinarian on all study animals prior to initiation and on all main study animals prior to their respective necropsy.
- Clinical observations will be performed at least twice-daily for main study animals during the dosing phase. Mortality observations will be performed twice daily for toxicokinetic study animals

- Body weights will be recorded once prior to treatment and weekly thereafter. Terminal body weights will be recorded for main study animals prior to necropsy following an overnight fast.
- Dosing solutions will be prepared once prior to study initiation, homogeneity assays (suspensions) and entrance assays will be performed on each dose level prior to its use. Samples will be collected and sent to a lab of the sponsor's choice for analysis. **(Cost of sample analysis not included)**
- Blood samples from the systemic exposure group will be collected from 3/rats/sex/group in Groups 2-4 only, on Days 1 and 28, at 6 time points. The samples will be spun down, the plasma separated, frozen and shipped to a lab of the sponsor's choice for analysis (N=216). **(Cost of sample analysis not included)**
- Terminal blood samples (hematology, serum chemistry, coagulation), will be collected from main study animals at their respective necropsy. At unscheduled necropsies of moribund animals, blood samples will be collected prior to euthanasia, when possible. BASi Evansville personnel will conduct the clinical pathology analyses.
- Following 4 cycles of dosing (1 time every four weeks for 16 weeks), all main study mice will be euthanized with carbon dioxide inhalation, exsanguinated and a gross necropsy performed. The remaining animals will be euthanized following a 4 week recovery period. A standard list of tissues will be collected and preserved in 10% NBF.
- All collected tissues from Groups 1 and 4, tissues from animals that die early, and any gross lesions will be embedded in paraffin, processed into blocks, sectioned, and stained. All slides from Group 1 & 4, slides from unscheduled deaths, and all gross lesions will be examined by a veterinary pathologist (ACVP). Target organs will be read in low and intermediate groups once identified and upon consultation with pathologist, study director and study monitor.
- An audited draft of the final report will be available 12 weeks following the final necropsy.
- The final report will be available 4 weeks after receipt of the sponsors edits to the audited draft and the applicable formulation analysis and TK reports generated from outside contract labs.

GLP 16-Week IV Toxicity Study in Cynomolgus Monkeys

- This study will be designed to conform to the FDA Good Laboratory Practice for Nonclinical Studies regulations (CFR 21 Part 58).
- The main study will consist of three dose groups and a control group of 3/sex/group in the main study and 2/sex/group in the control and high dose groups for recovery. Naïve cynomolgus monkeys (2-3 kgs), 30 days acclimation. N=32
- Blood samples will be collected from all treated animals (no controls) at each of 6 time points on days 1 and 28. Samples will be spun down, the plasma separated, frozen and shipped to the sponsor for analyses. N=384 plasma samples. **(Cost of sample analysis not included)**
- Dosing solutions will be prepared once prior to study initiation, homogeneity assays (suspensions) and entrance assays will be performed on each dose level prior to its use. Samples will be collected and sent to a lab of the sponsor's choice for analysis. **(Cost of sample analysis not included)**

- Food and water will be available *ad libitum*
- Body weight weekly
- Feed consumption daily
- Clinical observations twice-daily.
- Ophthalmology on all study animals prior to initiation and near the end of the dosing and recovery phase.
- ECGs recorded for all study animals prior to initiation and near the end of the dosing and recovery phases. N = 72 ECGs
- Hematology, serum chemistry, and coags pretest and near the end of the dosing and recovery phases. N=72 sets of each. BASi Evansville personnel will conduct the clinical pathology analyses.
- Following 4 cycles of dosing (1 time every four weeks for 16 weeks), the first 3 monkeys/sex/group will be euthanized with sodium pentobarbital, exsanguinated and a gross necropsy performed. The remaining monkeys will be euthanized following a 4 week recovery period. A standard list of tissues (~ 66/animal) will be collected and preserved in 10% NBF. N=2112 tissues.
- Tissues to be weighed include adrenals, brain, epididymides, heart, kidneys, liver, lungs, ovaries, spleen, thyroid/parathyroid, testes and uterus.
- All collected tissues will be trimmed, embedded in paraffin, sectioned, slides prepared and examined by a veterinary pathologist (ACVP). N=2112 tissues for examination.
- An audited draft of the final report will be available 12 weeks following the final necropsy.
- The final report will be available 4 weeks after receipt of the sponsors edits to the audited draft and the applicable formulation analysis and TK reports generated from outside contract labs.

I would like to thank you for considering BASi as your CRO partner for development of new drug candidate. If you have any questions, please do not hesitate to call me.

Very truly yours,

John Devine, DABT
Vice President, Non-Clinical Services
BASi Evansville

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Seattle, WA 98104
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SWEDISH
CANCER INSTITUTE

September 10, 2013

Dear Drs. Lieber and Drescher,

I have reviewed your Ovarian Cancer SPORE proposal entitled "A combination therapy of JO-1 and PEGylated liposomal doxorubicin in ovarian cancer patients." On behalf of the Swedish Cancer Institute (SCI), I would be pleased to collaborate with you on this proposal. In particular, the Cancer Institute will identify and refer eligible ovarian cancer patients and assist you in the conduct of the Phase I clinical trial proposed in study aim 2 that will take place in years 3 through 5 of the SPORE. Dr. Gary Goodman will serve as the SCI Principal Investigator for this work, donating 5% FTE towards oversight of trial activities performed at Swedish.

Thomas D. Brown, MD, MBA
Executive Director
James R. Yates, MSPH, MBA, FACHE
Vice President, Operations

I understand that the primary goal of this trial is to confirm the feasibility and safety of JO-1 when administered in combination with Doxil in patients with progressive, persistent or recurrent ovarian/fallopian tube cancer who have received standard therapies. This is in line with SCI's desire to provide our patients with access to new and innovative research protocols.

SCI is one of the leading clinical trial sites in the western United States with more than 100 available studies at any given time, including 7 currently open ovarian cancer trials. SCI facilities encompass over 225,000 square feet, including 7,000 square feet dedicated to research offices and activity space. Our oncologists have extensive experience with treatment protocols and actively enroll their patients onto a variety of trials sponsored by SWOG, Puget Sound Oncology Consortium (PSOC), American College of Surgeons of Oncology Group and pharmaceutical companies.

Our collaboration on this proposed work will further the SCI's and POCRC's shared goal of fostering community-based solid tumor research in Seattle, of mutual benefit to both institutions. I wish you continued success in your research endeavors.

Sincerely,

Thomas D. Brown, M.D., MBA
Executive Director
Swedish Cancer Institute

J. RESOURCE SHARING PLAN(S)

We will follow NIH Grants Policies concerning the sharing of research data. As outlined by the NIH, we will make available to the public results generated by this project and any accompanying data that were supported by the NIH. We will consult the following NIH source for guidance on sharing data: Sharing of Research Data as defined in the "NIH Statement on Sharing of Research Data" February, 2003 and referenced online at http://grants.nih.gov/grants/guide/notice-files/NOT-OD-03-032.html. Major findings will be disseminated through articles in scientific publications. Publicly available files will have all potential identifiers removed in order to protect the rights and privacy of human subjects.